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TABLE OF CONTENTS

Vol XXIX, 1941

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No 1 (January 1941).

	PAGE
NARAYANAN, E K Some Observations on the Preparation of Mannose	1
DHARMENDRA and BOSE, R Complement-Fixation in Leprosy with Antigens prepared from various Acid-fast Bacilli	7
MAPLESTONE, P A., and DEY, N C Further Laboratory Tests on the Fungistatic and Fungicidal Effects of various Substances	23
RAO, S RAGHVENDER Rat-Fleas of Calcutta Investigated from a Point of View of Epidemiology of Plague (<i>with 1 Graph in text</i>)	51
KRISHNAMURTHY, P V, and GIRI, K V Further Studies on the Influence of Pyrophosphate on the Oxidation of Vitamin C (<i>with 2 Graphs in text</i>)	71
SWAMINATHAN, M The Effect of Washing and Cooking on the Nicotinic Acid Content of Raw and Parboiled Rice	83
PASSMORE, R, and SUNDARARAJAN, A R The Vitamin B ₁ Content of the Millets <i>Eleusine coracana</i> and <i>Sorghum vulgare</i> , Whole Wheat grown under Different Manurial Conditions, and Rice Stored Underground	89
MAJUMDAR, B N The Vitamin A Content of some Indian Fish-Liver Oils	95
BHAVE, P D Diet Surveys in the Central Provinces and Berar	99
BASU, K P, BASAK, M N, and DE, H N Studies in Human Nutrition Part III Protein, Calcium and Phosphorus Metabolism with Typical Indian Diets	105
KIRWAN, E O'G, SEN, K, and BISWAS, R B Nutrition and its Bearing on Preventable Blindness and Eye Diseases in Bengal Preliminary Report	119
GIRI, K. V, and NAGANNA, B An Adsorption Method for the Estimation of Nicotinic Acid Content of Foodstuffs	125
KOCHHAR, B D Nicotinic Acid in Blood	133

	PAGE
RAO, M V RADHAKRISHNA Pathological Changes occurring in the Parathyroids in Rats fed on a Poor Rice Diet (<i>with 3 Plates</i>)	137
MITRA, K Dietary and Physique of Mining Population in Jharia Coal Fields (Bihar)	143
LAL, R B, and DAS GUPTA, A C Investigations into the Epidemiology of Epidemic Dropsy Part X A Note on an Outbreak of Epidemic Dropsy associated with the Use of Mustard Oil pressed from Seeds adulterated with Seeds of <i>Argemone mexicana</i> (<i>with 1 Map in text and 1 Plate</i>)	157
LAL, R B, CHATTERJI, S R, AGARWALA, S P, and DAS GUPTA, A C Investigations into the Epidemiology of Epidemic Dropsy Part XI Biological Test of Specific Toxin in Samples of Oil (<i>with 11 Graphs in text and 3 Plates</i>)	167
CHOPRA, R N, CHOPRA, G S, and ROY, A C Urinary Excretion of Morphine in Opium Addicts with and without Lecithin-Glucose Treatment	195
GREVAL, S D S, CHANDRA, S N, and DAS, B C Complement-Fixation in Hydatid Disease Suggestions	203
RAO, S SUNDAR, and SUKHATME, P V Seasonal Variations in the Incidence of Filarial Lymphangitis (<i>with 11 Graphs in text</i>)	209
RAHMAN, S A, and ZAIDI, M A Study on the Normal Polynuclear (Arneth) Count at Hyderabad-Deccan (<i>with 2 Graphs in text</i>)	225
GREVAL, S D S, CHANDRA, S N, and WOODHEAD, L S F On Isohæmagglutination Nomenclature, Titration of Isohæmagglutinins Need for Revision of Technique of Grouping Blood, etc (<i>with 2 Plates</i>)	231
CHOPRA, R N, GANGULY, S C, and RAO, S SUNDAR Protein Fractions and other Physical Properties of Hydrocele Fluid	253

No. 2 (April 1941).

MENON, K P <i>Staphylococci</i> in Vaccine Lymph	259
BOSE, A N, DAS GUPTA, S J, and BASU, U P Activity of 2-Sulphanil-amido-4-methylthiazole against Type I Pneumococcus Infections in Mice A Preliminary Note	265
DE, S P, DATTA, S K, and CHATTERJEE, S C Bacteriostatic Effect of Human Bile after Oral Administration of Hexamine and Sulphanilamide as studied in a Patient with Biliary Fistula	271
NIYOGI, S P, PATWARDHAN, V N, ACHARYA, B N, and CHITRE, R G Balanced Diets Part II Studies on the Nutritive Value of Fish	279
NIYOGI, S P, PATWARDHAN, V N, and SIRSAT, M V Studies on Basal Metabolism in Bombay Part III An Examination of the Factors Influencing the Basal Metabolism (<i>with 1 Graph in text</i>)	287

Table of Contents

	PAGE
VEERARAGHAVAN N Elimination of Excess Nerve Tissue from Antirabic Vaccine	303
DOGRA J R Studies on Peptic Uleer in South India Part III Experimental Production of Gastro-duodenal Uleer	311
MITRA, K, and MITTRA, H C Estimation of the Proximate Principles of Food in a few Edibles by Chemical Methods	315
SWAMINATHAN, M Further Studies on the Cyanogen Bromide Method of Estimating Nicotinic Acid in Biological Materials (<i>with 1 Graph in text</i>)	325
KOCHHAR, B D Nicotinic Acid in Blood and in Urine	341
RAJAGOPAL, K Dark-Adaptation Tests in Cases of Clinical Night-Blindness (<i>with 1 Diagram and 3 Graphs in text</i>)	351
MUKHERJI, S P, LAL, R B, and MATHUR, K B L Investigations into the Epidemiology of Epidemic Dropsy Part XII Isolation of Active Substances from Toxic Oils (<i>with 1 Plate</i>)	361
GHOSH, B N, DE, S S, and CHAUDHURI, D K Separation of the Neurotoxin from the Crude Cobra Venom and Study of the Action of a Number of Reducing Agents on it	367
NAPIER, L EVERARD, NEAL-EDWARDS, M I and DAS GUPTA, C R Hematological Studies in Indians Part XIII Normal Indian Women in Calcutta (<i>with 1 Graph in text</i>)	375
MENON, T BHASKARA, and RAMAMURTI B The Behaviour of the Infective Larvæ of <i>Wuchereria bancrofti</i> with Special Reference to their Mode of Escape and Penetration of Skin (<i>with 1 Plate</i>)	393

No 3 (July 1941)

READ, W D B, and PANDIT S R Distribution of <i>V. cholera</i> and El Tor Type Strains in certain Rural Areas in India (<i>with 2 Diagrams in text</i>)	403
VENKATRAMAN K V KRISHNASWAMI A K and RAMAKRISHNAN C S Occurrence of <i>Vibrio</i> El Tor in Natural Sources of Water in the Absence of Cholera	419
LAL, R B RAJA K C K E, and SATYA SWAROOP Statistical Inquiry into the Epidemiology of Cholera in Bengal Part I A General Review of the Epidemiological Features of Cholera in Different Parts of Bengal (<i>with 4 Maps in text</i>)	425
LAL, R B, RAJA K C K E SATYA SWAROOP and BASAK K C Statistical Inquiry into the Epidemiology of Cholera in Bengal Part II Formation of Homogeneous Cholera Districts (<i>with 5 Maps in text</i>)	441

	PAGE
SATYA SWAROOP, RAJA, K C K E, LAL, R B, and BASAK, K C Statistical Inquiry into the Epidemiology of Cholera in Bengal Part III Endemicity and Epidemicity of the Homogeneous Cholera Districts (<i>with 1 Map in text</i>)	465
CHATTERJEE, S K, and MITTER, K N Hæmolytic <i>Streptococci</i> in the Throat of apparently healthy Persons Its Incidence and Causal Relationship to Puerperal Infection	483
CHATTERJEE, S K, and MITTER, K N Significance of Hæmolytic <i>Streptococci</i> in Parturient Women (<i>with 1 Graph in text</i>)	491
SATYA SWAROOP A Modification of the Routine Dilution Tests and Tables showing the Most Probable Number of Organisms and the Standard Error of this Number ..	499
SATYA SWAROOP A Consideration of the Accuracy of Estimation of the Most Probable Number of Organisms by Dilution Test (<i>with 2 Graphs in text</i>)	511
DHARMENDRA Complement-Fixation by Leprous Sera after Absorption by various Acid-fast Bacilli .	523
GREVAL, S D S, DAS, B C, and SEN GUPTA, P C Preparation and Use of the Witebsky, Klingenstein and Kuhn (W K K) Antigen	527
DHURANDHAR, C B The Reliability of Guinea-pig Inoculation Test for the Diagnosis of Human Tubercular Affections (<i>with 1 Chart in text</i>)	531
KRISHNAN K V, and NARAYANAN, E K Preparation of Peptone for Bacteriological Work (<i>with 2 Text-figures</i>)	541
GREVAL, S D S, and CHANDRA, S N An Important Antigenic Difference between Hæmagglutinogens M and N	547
AYKROYD W R and KRISHNAN, B G Rice Diets and Beriberi	551
SWAMINATHAN M Urinary Excretion of Vitamin B ₆ by Rats	557
SWAMINATHAN, M A Method for the Estimation of Vitamin B ₆ in Urine	561
SUNDARARAJAN, A R The Vitamin B ₁ Content of Human Milk	567
RAJAGOPAL, K Physical and Chemical Methods of Estimating Vitamin A in Shark and Saw-fish Liver Oils	575
GIRI, K V, and NAGANNA, B An Adsorption Method for the Estimation of Nicotinic Acid Content of Animal Tissues and Blood	585
BASU, N M and DE, N K Assessment of Vitamin A Deficiency amongst Bengalees and Determination of the Minimal and Optimal Requirements of Vitamin A by a Simplified Method for Measuring Visual Adaptation in the Dark (<i>with 5 Graphs in text</i>)	591
FABISCH, WALTER, and HAMBURGER, H J Observations on the Physical Development of Punjabi Boys (<i>with 6 Graphs in text</i>)	613

Table of Contents

ix

	PAGE
GOKHALE, S K Blood Urea Clearance in Normal Indians A Study based on the Examination of 110 Normal Indian Men	627
GHOSE, R, and MUKERJI, B Differences in the Rate of Chloral Clearance in Blood in Normal and Liver-damaged Dogs (<i>with 2 Graphs in text</i>)	639
SEHRA, K B, CHOPRA, I C, and MUKERJI, B Experimental Liver and Biliary Damage and Serum Phosphatase (<i>with 3 Graphs in text</i>)	647
IYENGAR, N K. Competition of Protein Substrates towards Proteolytic Enzymes	655
RAHMAN, S A Alterations in the Electrocardiographic Features brought about by Digitalis (<i>with 1 Plate</i>)	659
DOGRA, J R Studies on Peptic Ulcer in South India Part IV Incidence of Peptic Ulcer in India with Particular Reference to South India (<i>with 1 Map in text</i>)	665
IYENGAR, M O T Occurrence of <i>Wuchereria bancrofti</i> Infection in a Rural Area	677

No 4 (October 1941)

VENKATRAMAN, K V, and RAMAKRISHNAN, C S A Preserving Medium for the Transmission of Specimens for the Isolation of <i>Vibrio cholerae</i>	681
LAHIRI M N On the Foetal Infection by <i>L. icterohæmorrhagiae</i> in a Rat	685
BASU, P N, and SEN, S N A Peptic Digest Broth for the Formation of <i>Clostridium tetani</i> Toxin	689
GHOSH, L M, and MAPLESTONE, P A An Improved Method of Growing Pure Cultures of Ringworm Fungi (<i>with 1 Plate</i>)	691
RANGANATHAN, S Calcium Intake and Fluorine Poisoning in Rats	693
RANGANATHAN, S The Vitamin D Content of some Fish Oils	699
AYKROYD, W R, and KRISHNAN B G Infantile Mortality in the Beriberi Area of the Madras Presidency (<i>with 1 Chart in text</i>)	703
SHOURIE, K L Dental Caries in Indian Children	709
TELANG, D M, and BHAGWAT, G A Studies in the Vital Capacity of Bombay Medical Students Part I Statistical Correlation with Physical Measurements (<i>with 25 Graphs in text</i>)	723
DATTA N C Metallic Contamination of Foodstuffs Part IV Investigations on Contamination of Foodstuffs with Copper from Brass and Bronze Vessels and the Metabolic Experiments on the Absorption and Excretion of Copper in Rats	751
CHOPRA, RAM NATH, and CHAKRAVARTI, M A Preliminary Note on the Pharmacological Action of the Alkaloids of <i>Rauwolfia serpentina</i>	765

	PAGE
CHOPRA, RAM NATH, GUPTA, J C, and CHOPRA, G S Pharmacological Action of the Essential Oil of <i>Curcuma longa</i>	769
ROY, A C, and CHOPRA, RAM NATH Lecithin and Hæmolysis	773
SMITH, R O A, HALDER, K C, and AHMED, I Further Investigations on the Transmission of Kala-azar Part IV The Duration of Life and other Observations on 'Blocked' Flies (with 2 Text-figures)	783
SMITH, R O A, and AHMED, I Further Investigations on the Transmission of Kala-azar Part V An Inquiry into the Relation between Malaria and Kala-azar in a Rural Area (with 1 Graph in text)	789
SMITH, R O A, HALDER, K C, and AHMED, I Further Investigations on the Transmission of Kala-azar Part VI A Second Series of Transmissions of <i>L donovani</i> by <i>P argentipes</i>	799
ADLER, S, and BER, M The Transmission of <i>Leishmania tropica</i> by the Bite of <i>Phlebotomus papatasi</i> (with 1 Plate)	803
FRENKEL, J Note on an Intracellular Stage of <i>Leishmania chameleons</i> , Wenyon 1921 (with 2 Text-figures)	811
LAL, R B, DAS GUPTA, A C, AGARWALA, S P, and ADAK, B Investigations into the Epidemiology of Epidemic Dropsy Part XIII Application of the Biological Test to Modified Argemone Oil and its Derivatives (with 16 Graphs in text)	813
LAL, R B, DAS GUPTA, A C, MUKHERJI, S P, and ADAK, B Investigations into the Epidemiology of Epidemic Dropsy Part XIV Feeding Experiments on Human Subjects to test the Toxicity of some of the Derivatives and Modifications of Argemone Oil	839
JOB, T J Food and Feeding Habits of the Glassfishes (<i>Ambassis Cuv & Val</i>) and their Bearing on the Biological Control of Guinea-worm and Malaria (with 2 Text-figures)	851
STRICKLAND, C, and ROY, D N Myiasis-producing Diptera in Man	863
DYER, BRIAN R Studies of Ground Water Pollution in an Alkaline Alluvium Soil (with 1 Map, 3 Graphs and 1 Diagram in text, and 1 Plate)	867
DYER, BRIAN R Sodium Chloride as an Indicator for the Determination of the Flow of Soil Water	891
NAPIER, L EVERARD, SEN GUPTA, P C, and CHANDRA SEKAR, C Hæmatological Studies in Indians Part XIV The Measurement of the Red Cell Diameter Standard Price-Jones' Curve for an Indian Population (with 1 Text-figure and 1 Graph in text)	903
INDEX OF AUTHORS	917
INDEX OF SUBJECTS	927

SOME OBSERVATIONS ON THE PREPARATION OF MANNOSE

BY

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THE preparation of mannose by the acid hydrolysis of the endosperm of the vegetable ivory nut, the Brazilian Tegua palm, *Phytalepas macrocarpa*, has become a routine procedure in this laboratory. The material for this preparation is imported from America as a meal consisting of the shavings and filings produced as a by-product in button industry. The use of this meal in the preparation of mannose dates from Fischer (1888) and the method has been modified by Horton (1921) and by Clark (1922). Bose (1939) has followed the modified method and has shown that the impure solution of mannose produced therein as a half-way stage can be substituted for the crystalline sugar in bacteriological work connected with cholera.

In following this method the present author has found that during the recommended period of 2½ hours' hydrolysis in boiling normal sulphuric acid the yield of the sugar in the hydrolysate is very much lower than what was obtained by Bose (*loc cit*), and that this period is insufficient to liberate all the sugar from the polysaccharide. The hydrolysis has been repeated as many as 18 times (ten times on one sample of the meal and eight times with another) on two consignments of the meal imported on separate occasions and the digestibility as well as the total sugar content have been found to be of the same order in both cases. As a preliminary to devising more favourable conditions for the preparation, a comparative study of the rates of liberation of mannose from the meal by various methods of hydrolysis was undertaken as also control experiments on the effect of the drastic treatments on the liberated mannose. Since we are stocking our mannose in the form of a neutral, sterilized aqueous solution and as some of our older stock solutions were found to contain much less sugar than recorded on them, the stability of mannose in solution during storage was also investigated.

Figures for the comparative rates of liberation of mannose in various methods of hydrolysis are given in Table I. In Table II are given the actual yields of the sugar in the different preparations as against the conditions of the hydrolysis. The yields are expressed as grammes of sugar in 100 c c of the hydrolysate under comparable conditions of experiment. The figures for the stability of the sugar under the conditions of the hydrolysis as well as during storage are also given under separate headings.

Mannose has invariably been estimated gravimetrically as its phenyl-hydrazone and data have been collected to indicate the accuracy of this method. The identity of the sugar has been checked by the melting point of the phenyl-hydrazone derivative determined directly as well as after admixture with the derivative from an authentic sample.

TABLE I

Comparison of the rates of hydrolysis in the autoclave (10 lb), on the wire-gauze, and on the salt water-bath (105°C)

[Two hundred g of the meal treated to an alkaline wash, washed with water till neutral and dried. The resulting powdered meal which is 75 per cent of the original weight is mixed with 110 c c of 65 per cent (13 N) sulphuric acid and left at room temperature for 24 hours. Then are added 1,200 c c of water and the meal suitably hydrolysed. Portions of the digest taken out periodically, filtered and estimated for mannose.]

Time in hours	PER CENT OF MANNOSE IN THE HYDROLYSATE		
	Autoclave (10 lb)	Wire-gauze	Salt water bath
0			
1		2.9	2.4
2		4.0	3.3
2½	4.2		
3		5.1	4.8
4		5.4	4.7
7		5.8	4.9
10		6.2	
15			6.2

TABLE II

Yields of mannose in different preparations

Ivory nut meal used	Process of hydrolysis	Duration of hydrolysis in hours	Number of times hydrolysis was repeated in the same way	Average yield, per cent
Meal 1	Autoclave 10 lb	2½	1	4.2
	Wire gauze	4	1	3.9
	Salt water bath, 105°C	2½	1	2.8
		5	2	3.4
		6	1	4.2
		7	3	4.5
Meal 2	Wire gauze	10	1	6.2
	Salt water bath, 105°C	7	1	4.2
		9	3	5.0
		15	3	6.3

Degree of accuracy in the estimation of mannose as phenyl-hydrazone

Experiment 1—Solutions of crystalline mannose were made and immediately analysed

Concentration of the solution by actual weight of sugar, per cent	Concentration found by analysis, per cent
2	1.8
4	3.8

Experiment 2—Two mannose preparations were analysed in duplicate

Number of preparation	Analysis No 1, per cent	Analysis No 2, per cent
N6	4.93	4.88
N7	4.34	4.50

Destruction of mannose by contact with N acid H_2SO_4

Experiment 1—A 6 per cent solution of crystalline mannose in N sulphuric acid was made and one portion kept at room temperature ($30^\circ C$) for two days and analysed

Concentration found

5.47 per cent

In view of the fact that a 2 per cent solution of crystalline mannose was found to be 1.8 per cent and a 4 per cent mannose was found to be 3.8 per cent on immediate analysis the above figures for contact with N acid for two days indicate no destruction of sugar

Experiment 2—Another portion of 6 per cent mannose in N sulphuric acid was boiled under reflux for ten hours and the volume checked and found to be unchanged. The solution was then analysed

Concentration found

5.1 per cent

Based on the value of 5.47 per cent estimated for the unboiled sample there is a reduction of 7 per cent in the sugar concentration

Stability of mannose in neutral (pH 6 to 7) sterilized aqueous solutions at summer temperatures ($35^\circ C$) for periods nearly three months

Solution number	Concentration at the beginning of the period, per cent	Concentration after the lapse of 3 months, per cent
N6	4.35	4.43*
N2	2.8	2.65

* Note the degree of accuracy usually observed in duplicate determinations

It will be seen that with the two meals under consideration about 4 per cent mannose accumulates in the hydrolysate during $2\frac{1}{2}$ hours' direct boiling on the wire-gauze or autoclaving at 10-lb pressure, and a little less than this amount results by digestion in a boiling saturated salt water-bath ($105^\circ C$). Bose has, however, obtained a yield of 8.5 per cent sugar in the hydrolysate during the same period by working on a sand-bath. Comparatively the wire-gauze process is liable to breakage of the glass apparatus due to the almost unavoidable violent bumping of the saw-dust-like material which is never completely dissolved. Autoclaving is simple enough, but the product is caramelized. Digestion on the salt water-bath is slower but safer. Some discontinuity observable in the course of the hydrolysis in this case is due to the unsteadiness in the heating qualities of our improvised baths. The maximum yield of sugar thus far obtained is about 6 per cent in solution and this occurs after ten hours' digestion over the wire-gauze or 15 hours in the salt water-bath. Control experiments for the destruction of

mannose under the conditions of their prolonged hydrolysis showed with crystalline mannose about 7 per cent decomposition in 10 hours. This loss, however, is more than compensated for by the increased yields. No deterioration of sugar takes place by contact with normal sulphuric acid at ordinary room temperatures (30°C) for periods as long as two days and so there is no necessity to hurry through the different stages of the preparation. Neutral (pH between 6 and 7) sterilized aqueous solutions of mannose prepared in this manner have remained undiminished in strength at summer temperatures in Bengal (35°C) for periods as long as three months. This fact has been particularly helpful in clearing certain doubts which cropped up as a result of some of our older stock mannose solutions being found to be much poorer in strength than indicated on their labels.

In carrying out the hydrolyses as well as in estimating the mannose no deviations have been made from previous workers in essential details. It may, however, be said in passing that in the removal of sulphuric acid from solutions of mannose barium carbonate is to be preferred to barium hydroxide as the danger of a large local increase in the alkalinity of the hot solution, resulting in a certain amount, however small, of destruction of sugar and its mutation to fructose and glucose (Jobry de Bruyn and van Ekenstein transformation) is absent in the case of the carbonate. It is at the same time cheaper.

For the quick and quantitative removal of barium from mannose solutions a simple and rapid technique has been developed. An aliquot portion, usually 2 c.c. of the solution, is treated in a small centrifuge tube with small quantities at a time, as in a titration, of a dilute solution of sulphuric acid of known strength from a microburette, and every time the precipitate is sedimented in the same tube with the help of a hand centrifuge. The exact quantity of sulphuric acid needed is indicated by the point at which no precipitate forms when a drop of acid is added to the clear supernatant. Knowing the titre value of the aliquot the amount for the whole of the liquid is calculated in terms of a more concentrated acid and this quantity advantageously added without unduly diluting the fluid. This technique has been followed with uniform success in all the preparations and the resulting solutions have been found to be free from barium and all but a trace of sulphate.

SUMMARY

1 The usually recommended period of $2\frac{1}{2}$ hours' hydrolysis in boiling normal sulphuric acid is insufficient to liberate all the available mannose from its polysaccharide in the ivory-nut meal, while about ten hours' direct boiling on the wire gauze is found to be necessary.

2 The amount of destruction of sugar by contact with boiling normal sulphuric acid for ten hours is about 7 per cent, while there is no reduction in the concentration of the sugar if the acid solution is kept at room temperature (30°C) for two days.

3 Comparative studies have been made on different methods of conducting the hydrolysis of the ivory-nut meal and digesting in the salt water-bath (105°C) is recommended as the safest and the best.

4 Mannose as obtained in these hydrolyses has been found to remain undiminished in strength when kept in a neutral (pH 6 to 7) sterilized solution for periods as long as three months at temperatures near about 35°C

5 A simple and rapid technique has been developed for the removal of barium and inferentially of sulphate from solutions

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COMPLEMENT-FIXATION IN LEPROSY WITH ANTIGENS PREPARED FROM VARIOUS ACID-FAST BACILLI

BY

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AND

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INTRODUCTION

LOWE AND GREAV (1939) briefly reviewed the literature of complement-fixation in leprosy with special reference to the use of Witebsky, Klingenstein and Kuhn (W K K) antigen—an antigen prepared from the alcohol-insoluble portion of the human tubercle bacillus. Using this antigen these authors performed the test in a large number of cases of leprosy and of other diseases. They considered the test to be of little value in diagnosis or prognosis of the disease. Moreover, the test was not found to be specific for leprosy since positive results could be obtained in other diseases, chiefly leishmaniasis, and since cases of the neural type of leprosy usually gave negative results.

All the workers who have studied complement-fixation in leprosy agree that a large percentage of cases of leprosy give a positive complement-fixation test with antigens prepared from the various acid-fast bacilli. The fixation of the complement by leprosy sera in presence of an antigen prepared from an organism isolated from leprosy lesions could not therefore mean that the organism in question was the causative organism of leprosy. Cooke (1919) who performed complement-fixation in 20 cases of leprosy using as antigen emulsions from 16 different acid-fast bacilli arrived at this conclusion. Similar findings were made by Lewis and Aronson (1923) who used 13 different bacillary antigens. Lleras Acosta (1936), however, reported that the results of complement-fixation by leprosy sera with an antigen prepared from the acid-fast bacillus which he had isolated from the blood of lepers furnished a 'proof of unquestionable value' that the organism isolated by him was the genuine organism of leprosy.

The work of Cooke and of Lewis and Aronson was done with crude antigens. Since then efforts have been made to improve the antigens in order to make complement-fixation more specific. It appeared possible, though not very likely, that with the antigens prepared according to recent methods and with the improved technique, complement-fixation tests might throw some light on the genuineness of the supposed cultures of the leprosy bacilli. The present investigation was undertaken with a view to exploring this possibility.

It was decided to test the complement-fixing power of sera from cases of leprosy and of other diseases in the presence of the antigens prepared from various acid-fast bacilli including some of the supposed cultures of leprosy bacilli. The antigens from the different cultures were to be prepared by the same method and the same technique for performing the test was to be used throughout. The technique used by Lowe and Greval (*loc cit*) and described by Greval, Lowe and Bose (1939) was selected as it takes into consideration the proper standardization of the antigen and the proper measurement of complement. The technique consists in using a standardized antigen and the hæmolytic system of method No. 4 of the Report of the Medical Research Committee on the Wassermann test.

SELECTION OF A METHOD FOR THE PREPARATION OF THE ANTIGENS

A number of preliminary tests was carried out to select a method for the preparation of an antigen likely to be the most specific and sensitive.

The two methods —The choice lay between the method of Witebsky, Klingenstein and Kuhn (W K K) and that of Boquet and Negret which was used by Lleras for the preparation of his antigen. The antigen prepared by the W K K method consists of a pyridin extract of the alcohol-insoluble portion of the bacilli, dissolved in benzol. The antigen prepared by the Boquet and Negret's method, however, consists of an alcoholic extract of the dried and powdered bacilli. Lecithin is added to the W K K antigen but not to the Boquet and Negret antigen. The addition of lecithin to an antigen is known to make it more sensitive. It was, therefore, considered desirable to find out first if lecithin will increase the sensitivity of the antigen prepared according to Boquet and Negret's method.

Sensitization of Lleras' antigen by the addition of lecithin —Lleras' antigen was sensitized by the addition of lecithin in the following manner. One c.c. of 1 per cent alcoholic solution of lecithin was evaporated and the residue was dissolved in 2 c.c. of the antigen. The antigen, after the addition of lecithin, was suitably diluted. Parallel tests using the sensitized and the unsensitized antigens were performed with sera from 14 cases of leprosy and eight cases of other skin diseases. The results with both the antigens were similar with the sera from cases of the 'lepromatous' type of leprosy and from cases of the other diseases. The addition of lecithin to the antigen, however, increased its sensitivity when tested with the sera from cases of the 'neural' type of leprosy. There were nine such cases in the series, with the unsensitized antigen there was complete hæmolysis in all the cases. With the sensitized antigen, however, there was complete or partial hæmolysis in only four cases, in the other five cases hæmolysis was inhibited completely or almost completely.

Comparison between the method of Boquet and Negret and that of W K K— Having observed that the addition of lecithin increased the sensitivity of an antigen prepared according to Boquet and Negret's method, the next step was to compare this sensitized antigen with the one prepared by the W K K method. For this purpose antigens were prepared from cultures of Kedrowsky's and Lleras' bacilli by the two different methods above mentioned and were sensitized by the addition of lecithin. These antigens were then tested against sera from cases of the 'neural' and the 'lepromatous' types of leprosy and other skin diseases. The results obtained with the two antigens prepared from the Kedrowsky's bacillus are shown below —

Disease	Number of cases	COMPLETE OR AT MOST COMPLETE INHIBITION OF HEMOLYSIS WITH THE ANTIGENS PREPARED BY	
		W K K method	Bouquet and Negret's method
<i>Leprosy—</i>			
'Lepromatous' type	20	19	17
'Neural' type, bacteriologically positive	14	11	9
'Neural' type, bacteriologically negative	52	13	13
TOTAL	86	43	39
<hr/>			
<i>Other diseases</i>	53	11	13

The antigens prepared from the Lleras' bacillus gave similar results

Thus, the antigen prepared according to the W K K method appears to be more sensitive than the one prepared by the method of Boquet and Negret. The W K K method was, therefore, selected for the preparation of the antigens from the different acid-fast bacilli to be used in our investigation.

COMPLEMENT-FIXATION WITH THE ANTIGENS FROM SIX DIFFERENT ACID-FAST BACILLI PREPARED BY THE W K K METHOD

The organisms used—The acid-fast bacteria used for the preparation of the antigens in the laboratory included cultures of *Mycobacterium phlei* and of the so called leprosy bacilli of Kedrowsky, Lleras, Beyon and Duval. The W K K antigen from tubercle bacillus was purchased ready made.

Standardization of the antigens—All the six antigens were titrated to find out the strongest dilutions which could be used without getting any anti-complementary effect. These dilutions were found to be between 1 in 40 and 1 in 60 for the different antigens. Each time a test was set up, a tube containing one dose of such antigen dilution was put up with one m h d of complement to detect any anti-complementary activity of the antigen. In the test proper two m h d of complement were used.

The sera used for the tests—Sera from 125 cases of leprosy and from 58 cases of other diseases have been tested. Results with the sera from 13 cases of leprosy had, however, to be ignored because of the discovery that after four months the titre of the antigens had fallen markedly. The findings made in 112 cases of leprosy and 58 of other diseases are, therefore, left.

The sera for the test were inactivated at about 55°C for 30 minutes. All the sera were first tested in a 1 in 5 dilution, for the sera which inhibited hæmolysis either completely or almost completely (with only a trace of lysis) further dilutions of 1 in 25, 1 in 50 and 1 in 100 were put up.

The results with the sera diluted 1 in 5—The detailed results of the tests with 1 in 5 dilutions of the sera are shown in Tables I, II and III. These results may be summarized as under—

Disease	Total number of cases	CASES PRODUCING COMPLETE INHIBITION OF LYSIS IN PRESENCE OF ALL THE SIX ANTIGENS		CASES PRODUCING COMPLETE INHIBITION OF LYSIS IN PRESENCE OF ONE OR MORE BUT NOT ALL THE ANTIGENS	
		Number	Percentage	Number	Percentage
<i>Leprosy—</i>					
‘Lepromatous’ type	19	16	84.2	2	10.5
‘Neural’ type, bacteriologically positive	13	8	61.5	5	28.5
‘Neural’ type, bacteriologically negative	80	18	22.5	6	7.5
<i>Other diseases—</i>					
Leishmania infections	8	8	100	0	0.0
All other diseases	50	0	0	4	8.0

A consideration of the results set out in Tables I, II and III (see *Appendix*) brings out the following points —

1. A very high percentage of sera (81·2 per cent) from cases of the 'lepromatous' type of leprosy fixed the complement completely in presence of all the six antigens. The percentage of such sera is lower in the bacteriologically positive cases of the 'neural' type (61·5 per cent) and much lower in the bacteriologically negative cases of the 'neural' type (22·5 per cent).

2. Out of the 58 sera from cases of other diseases only eight (13·7 per cent) fixed the complement completely in presence of all the antigens. Seven of these sera were obtained from patients suffering from kala-azar and the eighth one from a case of dermal leishmaniasis. If these cases of leishmania infection are excluded, not in a single instance did a serum from a patient suffering from any disease other than leprosy fix the complement completely in presence of all the antigens.

3. All the six antigens appear to behave in a similar way but the antigen prepared from *Lleris bacillus* appears to be slightly more sensitive than the other antigens. It will be seen from Table II that 15·8 per cent of all the sera fixed the complement completely or almost completely in presence of the antigen prepared from *Lleris bacillus*, while the percentage of the sera acting similarly in presence of the other antigens varied between 38·8 and 41·8.

This difference in the sensitivities of the different antigens is also brought out by analysis of the results of the sera which acted differently with the different antigens. Identical results were obtained with all the antigens in 140 of the sera tested, i.e. in 82·4 per cent of the sera. The findings made with the remaining 30 are shown in Table III. This table shows that complete or almost complete inhibition of lysis was seen 26 times in presence of the antigen from *Lleris bacillus* while with the other antigens the number of such reactions varied between 14 and 19.

4. This difference in the sensitivities of the antigens does not appear to be caused by any specificity. Tables II and III show that the greater sensitivity of a particular antigen is as apparent in the non-leprosy cases as in the leprosy cases. For example, let us consider the antigen prepared from *Lleras' bacillus* which has been found to be the most sensitive of all the antigens used. A reference to Table II will show that the higher sensitivity of this antigen is most apparent in the bacteriologically negative cases of the 'neural' type of leprosy and in diseases other than leprosy. If we take into consideration the individual cases, we find that the antigen prepared from *Lleras' bacillus* was found less sensitive than the other antigens in one case (leprosy) and more sensitive in nine (four leprosy and five leucoderma) cases.

Further dilutions of the sera — There were 82 sera (19 from cases of the 'lepromatous' type, 45 from cases of the 'neural' type of leprosy and 18 from cases of other diseases) which inhibited lysis completely or almost completely in 1 in 5 dilution with one or more antigens. The tests were then performed with 1 in 25, 1 in 50 and 1 in 100 dilutions of all these sera. The results of these tests

are shown in Table IV. It will be seen that the results with the diluted sera vary according to the source of the serum and to some extent according to the antigen used. These results can be summarized as under —

1 *Source of the serum* — In cases of the 'lepomatous' type of leprosy dilution of the serum to 1 in 25 has practically no effect on the result of the test, a dilution to 1 in 50 reduces the number of sera which fix the complement, a dilution to 1 in 100 further reduces the number with the result that only six to eight of the 19 sera fixed the complement in this dilution.

In the bacteriologically positive cases of the 'neural' type a dilution to 1 in 25 considerably reduces the number of reacting sera, a dilution to 1 in 50 further reduces this number, and in a dilution to 1 in 100 only two of the 13 sera fixed the complement and that also with one antigen only, that from Lleras' bacillus. Sera from the bacteriologically negative cases of the 'neural' type behaved in much the same way as those from the bacteriologically positive cases of the 'neural' type.

In the other diseases, with the exception of kala-azar, a dilution to 1 in 25 usually removed from the serum its complement-fixing property. Only two sera provided an exception to this. In kala-azar, however, even a dilution to 1 in 100 did not remove the complement-fixing property of the sera. There were seven sera from kala-azar in our series and all the seven fixed the complement with all the antigens in a 1 in 100 dilution.

In general we can say that with a serum diluted 1 in 25, complement-fixation is practically limited to cases of leprosy and kala-azar, but the adoption of this dilution as the standard would reduce markedly the reacting powers of the sera from the neural cases of leprosy.

2 *Nature of the antigen* — Another factor which effects complement-fixation with the different dilutions of the sera is the nature of the antigen.

As already stated, an analysis of the results with 1 in 5 dilutions of the sera showed that the antigen prepared from the Lleras' bacillus was slightly more sensitive than the other antigen. A consideration of the results of the tests with the different dilutions of the sera also leads one to the same conclusion. Table V shows the results of the tests with the different dilutions of the 60 sera which inhibited hæmolysis completely or almost completely in 1 in 5 dilution with all the six antigens used. The results shown in Table V may be summarized as under —

With sera from kala-azar cases all the antigens behave in an identical way.

In cases of the 'lepomatous' type, the antigen prepared from Lleras' bacillus appears to be slightly more sensitive than the other antigens.

It is in cases of the 'neural' type of leprosy—both bacteriologically positive and negative—that the greater sensitivity of this antigen is more clearly seen. Thirty-four sera from cases of the neural type of leprosy fixed the complement in 1 in 5 dilution with all the antigens. In 1 in 25, 1 in 50 and 1 in 100 dilutions the number of the sera that fixed the complement gradually fell. The extent of the fall, however, varied with the different antigens. In all the dilutions, the number

of sera which fixed complement was greatest in the presence of the antigen prepared from Lleras' bacillus as shown below —

Antigen prepared from	NUMBER OF SERA WHICH FIXED COMPLEMENT IN DILUTIONS OF		
	1 in 25	1 in 50	1 in 100
Lleras' bacillus	22	10	4
McCoy's bacillus	12	5	3
Duval's bacillus	11	4	2
Ravon's bacillus	10	4	1
McCoy's bacillus	9	1	1
Kedrowsky's bacillus	8	3	2

The higher sensitivity of the antigen prepared from Lleras' bacillus — Thus, of all the antigens used in our experiments, the one prepared from Lleras' bacillus has been found to be slightly more sensitive than others.

As has already been pointed out this greater sensitivity of the antigen prepared from Lleras' bacillus does not appear to be caused by any specificity. The view that this higher sensitivity is not caused by any specificity is based on the following facts —

- (i) The reaction itself is non specific. The property possessed by sera from cases of leprosy of fixing complement in presence of the antigens from various acid fast bacteria is possessed to an even more marked extent by sera from cases of kala-azar.
- (ii) The higher sensitivity of the antigen prepared from Lleras' bacillus is seen both in leprosy and non-leprosy cases.

The difference between the sensitivities of the antigens prepared from the different acid-fast bacilli has also been noted by some of the previous workers. Cooke (*loc cit*) found that the antigens prepared from the bacilli of Duval and of Kedrowsky and from the tubercle, and the smegma bacilli, were superior to the antigens prepared from other acid-fast bacilli. Similarly, Lewis and Aronson (*loc cit*) found that Clegg's bacillus produced a better antigen than the other acid-fast bacteria used. This superior antigenic property of certain bacilli was found to be associated with another property, that of making better suspensions in saline and it has been suggested that the former property may be dependent on the latter. In this connection we have found that Lleras' bacillus makes a better suspension in saline than the other acid-fast organisms included in our tests.

Although we believe that the higher sensitivity of the antigen from Lleras' bacillus is not caused by any specificity, the question is being further studied by means of the antibody absorption tests and by allergic tests in rabbits.

COMPLEMENT-FIXATION IN DISEASES OTHER THAN LEPROSY

In the preliminary tests, nine of the 53 sera from cases other than leprosy fixed complement in presence of the antigen prepared from Kedrowsky's

bacillus These findings were made twice in 'aldehyde-positive' sera, twice in Wassermann positive sera, and once each in cases of leucoderma, seborrhœa, lupus erythematosus, hyperkeratosis and actinomycosis

In the test proper 58 sera from cases other than leprosy were tested against all the six antigens The source of these sera and the results obtained are shown below —

Source	Number of sera	Number fixing the complement in 1 in 5 dilution in the presence of one or more antigens
Wassermann positive sera	14	3
Wassermann negative sera	2	1
Kala azar	7	7
Dermal leishmaniasis	1	1
Leucoderma	20	6
Other skin diseases	14	0
TOTAL	58	18

Further details about the findings made in these cases will be found in Tables I to V (see *Appendix*)

THE NATURE OF THE SUPPOSED CULTURES OF *Mycobacterium lepræ*

As already stated at the beginning of this paper certain workers have supported their claim to have cultured *Myc lepræ* by producing evidence that in the presence of an antigen prepared from that particular organism the serum of lepers fixed complement The work here recorded was undertaken in order to find out whether complement-fixation tests performed with the antigens prepared by modern methods from various organisms isolated by various workers from leprous lesions would give any evidence of the genuineness of these supposed cultures

The evidence here produced shows that (1) the antigens from the bacilli of Bayon, Duval, Lleras and Kedrowsky and from *Myc tuberculosis* and *Myc phlei* all act in a similar way, (2) this action is non-specific and (3) there are minor degrees of differences in the sensitivities of the different antigens It is, therefore, concluded that complement-fixation tests have not given, and are unlikely to give, any evidence either for or against the genuineness of cultures of organisms isolated from leprous lesions The proof of the genuineness of such a culture must be based on other grounds

SUMMARY.

1 The work was undertaken to find out whether complement-fixation tests done with sera from cases of leprosy and with antigen prepared by modern methods

from the so called cultures of leprosy would give any evidence regarding the relation of these isolated organisms to the disease

2 Complement fixation tests have been performed in 112 cases of leprosy and 58 cases of other diseases in presence of antigens prepared from six different acid fast bacilli including the so called leprosy bacilli of Duval, Bayon, Kedrowsky and Lleras. All the antigens were prepared by the method by Witebsky, Klingenstein and Kulin

3 Sera diluted 1 in 5 fixed complement in presence of all the six antigens, in 84 per cent of the 19 cases of the 'lepromatous' type, 61.5 per cent of the 13 bacteriologically positive cases of the 'neural' type and 22.5 per cent of the 80 bacteriologically negative cases of the 'neural' type and all the eight cases of leishmania infection

4 In 1 in 5 dilution 21 per cent of the 14 Wassermann positive sera and 30 per cent of the 20 sera from cases of leucoderma fixed complement in presence of one or more but not all the antigens

5 With sera diluted 1 in 25 complement-fixation is practically limited to cases of leprosy and kala-azar. In this dilution however the number of reacting sera of the 'neural' type of leprosy is reduced markedly

6 All the six antigens appear to behave in a similar way but the antigen prepared from the Lleras' bacillus appears to be slightly more sensitive. A slightly higher number of sera fix complement in its presence and dilution does not reduce complement-fixation with this antigen to the same extent as with the other antigens

7 This greater sensitivity of the antigen prepared from Lleras' bacillus does not appear to be caused by any specificity as it is seen both in leprosy and non-leprosy cases

8 It is concluded that complement-fixation tests have not given, and are unlikely to give, any evidence regarding the genuineness of cultures of organisms isolated from leprosy lesions

ACKNOWLEDGMENTS

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APPENDIX

TABLE I.

Results of complement-fixation tests in leprosy and other diseases by sera diluted 1 in 5 and with antigens prepared from six different acid-fast bacteria according to the method of W K K

Disease	RESULTS						In how many cases
	ANTIGENS PREPARED FROM						
	Tubercle bacillus	Kedrowsky's bacillus	Lleras' bacillus	<i>Myc phlei</i>	Bayon's bacillus	Duval's bacillus	
<i>Leprosy—</i> 'Lepromatous' type (19 cases)	N T H	N T H	N N T	N N H	N N H	N N H	16 2 1
<i>Leprosy—</i> 'Neural' type, bacteriologically positive (13 cases)	N T N T H	N N N T H T	N N T T N	N N N N H H	N N N N H H	N N N N H H	8 1 1 1 1 1
<i>Leprosy—</i> 'Neural' type, bacteriologically negative (80 cases)	N T N T H H H T H H	N N T N T N N H H H H	N N N N T N N T T T H	N N N T T N T T H H H	N N N T T N N T H H H	N N N T T N T H H H	18 2 1 1 2 1 1 1 3 2 48
<i>Other diseases</i> (58 cases)	N H H T T H H H	N T T T H H H H	N N H N N T H H	N N N H H H H	N N N T T H H H	N N N T T H T H	8 1 1 1 1 3 3 40

N = No hæmolysis (complete inhibition of lysis)

T = Traces of hæmolysis (almost complete inhibition of lysis)

H = Hæmolysis, complete or partial (more than a trace of lysis).

TABLE II.

An analysis of the results of complement fixation tests in leprosy and other diseases by serial dilution in 5 and with the different antigens,

Disease,	Number of cases	Cases which remained in solution completely or almost completely with the antigen prepared from									
		Tubercle bacillus,		Kodrowsky's bacillus,		Léon's bacillus,		Myc. phlei,		Hayon's bacillus,	
		Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent
Leprosy—	• Lepromatous, type . . .	10	18	01.7	18	01.7	100.0	18	01.7	18	01.7
	• Nodul. type, bacteriologically pos. live,	13	12	02.3	13	02.3	100.0	11	81.0	11	81.0
	• Nodul. type, bacteriologically neg. live,	80	27	33.7	28	35.1	10.0	27	33.7	27	33.7
TOTAL . . .		111	57	50.8	50	40.0	01	50	10.0	57	51.0
Other diseases . . .	• . . .	58	10	17.2	11	10.0	14	10	17.2	12	20.0
	GRAND TOTAL . . .	170	67	39.1	70	41.2	78	60	38.8	60	35.3

TABLE III.

An analysis of the results in the 30 sera in which the different antigens gave different results

Disease	Number of cases	NUMBER SHOWING COMPLETE OR ALMOST COMPLETE INHIBITION OF HÆMOLYSIS WITH THE ANTIGENS PREPARED FROM					
		Tubercle bacillus	Kedrowsky's bacillus	Lleras' bacillus	<i>Myc. phlei</i>	Bayon's bacillus	Duval's bacillus
<i>Leprosy—</i>							
‘Lepromatous’ type	3	2	2	3	2	2	2
‘Neural’ type, bacteriologically positive	5	4	4	5	3	3	3
‘Neural’ type, bacteriologically negative	12	7	6	12	7	7	7
TOTAL	20	13	12	20	12	12	12
<i>Other diseases</i>	10	2	3	6	2	4	7
GRAND TOTAL	30	15	15	26	14	16	19

TABLE IV

An analysis of the results of complement-fixation tests with the different dilutions of the S2 sera which inhibited lysis completely or almost completely in 1 in 5 dilution

Disease	Number of cases	Serum dilution	NUMBER PRODUCING COMPLETE OR ALMOST COMPLETE INHIBITION OF LYSIS WITH THE ANTIGENS PREPARED FROM					
			Tubercle bacillus	Kodrowsky's bacillus	Liloes' bacillus	<i>Myc. phlei</i>	Bayon's bacillus	Duval's bacillus
<i>L. procy-</i> 'Leptomatous' type	19	$\frac{1}{5}$	18	18	19	18	18	18
		$\frac{1}{25}$	17	17	18	16	17	17
		$\frac{1}{50}$	13	10	16	11	11	10
		$\frac{1}{100}$	7	6	8	7	7	7
		$\frac{1}{5}$	11	12	13	11	11	11
<i>L. procy-</i> 'Neural' type, bacteriologically positive	13	$\frac{1}{25}$	4	2	8	5	3	4
		$\frac{1}{50}$	0	1	4	1	0	0
		$\frac{1}{100}$	0	0	2	0	0	0
		$\frac{1}{5}$	27	26	32	26	27	26
		$\frac{1}{25}$	5	6	16	7	7	6
<i>L. procy-</i> 'Neural' type, bacteriologically negative	32	$\frac{1}{50}$	0	2	6	4	4	4
		$\frac{1}{100}$	0	1	2	3	1	2

Complement-Fixation in Leprosy

TABLE IV—*contd.*

Disease	Number of cases	Serum dilution	NUMBER PRODUCING COMPLETE OR ALMOST COMPLETE INHIBITION OF LYSIS WITH THE ANTIGENS PREPARED FROM					
			Tubercle bacillus	Kodrowsky's bacillus	Lieres' bacillus	<i>Myc phlei</i>	Bayon's bacillus	Duval's bacillus
<i>Kala-azar</i>	7	$\frac{1}{5}$	7	7	7	7	7	7
		$\frac{1}{25}$	7	7	7	7	7	7
		$\frac{1}{50}$	7	7	7	7	7	7
		$\frac{1}{100}$	7	7	7	7	7	7
<i>Other diseases</i>	11	$\frac{1}{5}$	3	4	5	3	5	8
		$\frac{1}{25}$	0	2	0	1	1	1
		$\frac{1}{50}$	0	1	0	1	1	1
		$\frac{1}{100}$	0	0	0	0	0	1

TABLE V

An analysis of the results of complement-fixation tests with the different dilutions of the 60 sera which exhibited lysis completely or almost completely in 1 in 5 dilution with all the six antigens used

NUMBER OF SERA PRODUCING INHIBITION OF HEMOLYSIS IN THE DIFFERENT DILUTIONS WITH THE VARIOUS ANTIGENS														
	LEPROSY												Dermal leish- maniasis	
	'Lepromatous', type			'Neural' type, bacteriologically positive			'Neural' type, bacteriologically negative			Kala azar				
	Dilutions			Dilutions			Dilutions			Dilutions				
Antigens prepared from	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	5	25	50	100	5	25	50	100	5	25	50	100	5	25
Tubercle bacillus	18	17	13	7	11	4			23	5	1	1	7	1
	18	17	10	6	11	2	2		23	6	2	2	7	1
	18	17	16	8	11	7	4	2	23	15	6	2	7	1
	18	18	11	7	11	5	1		23	7	4	3	7	1
Kedrowsky's bacillus	18	16	11	7	11	3			23	7	4	1	7	1
	18	17	10	7	11	4			23	7	4	2	7	1
Lleras' bacillus	18	17	16	8	11	7	4	2	23	15	6	2	7	1
	18	18	11	7	11	5	1		23	7	4	3	7	1
Myc phla	18	16	11	7	11	3			23	7	4	1	7	1
	18	17	10	7	11	4			23	7	4	2	7	1
Bayon's bacillus	18	17	13	7	11	4			23	5	1	1	7	1
	18	17	10	6	11	2	2		23	6	2	2	7	1
Duval's bacillus	18	17	16	8	11	7	4	2	23	15	6	2	7	1
	18	18	11	7	11	5	1		23	7	4	3	7	1

FURTHER LABORATORY TESTS ON THE FUNGISTATIC AND FUNGICIDAL EFFECTS OF VARIOUS SUBSTANCES

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MAPLESTONE AND DEY (1938) published a preliminary report on the relative efficiency of certain chemicals and dyes used in varying dilutions against four species of fungi common in Calcutta (*Epidermophyton floccosum*, *Achorion violaceum*, *Microsporum audouinii* and *Achorion actoni*) and a strain of *Staphylococcus aureus* which is a common complicating infection with that of *A. violaceum*. In most instances the dilution which effected inhibition of growth or death of the organism and the next higher dilution, which failed to do so, had rather wide gaps. The purpose of the present paper is to show the effective end-points of the same substances in closer ranges of dilution utilizing the same organisms. In the present instance a mixture of gentian violet and brilliant green has been added to the series, thus increasing the number of test substances from 19 to 20.

TECHNIQUE

The technique employed on this occasion was exactly the same as in the first series of tests except that the standard emulsion of the test culture was used for our fungistatic as well as for our fungicidal tests. The advantages of using a standard emulsion of the test culture for inoculation over that of

transferring direct a small portion of the culture itself, may be enumerated as follows —

- (a) The size of the inoculum picked up cannot be determined with sufficient accuracy to ensure that comparable amounts are used in all the test cultures
- (b) The small amount of pure Sabouraud medium that is inadvertently transferred with the inoculum aids its initial growth on the new medium before it comes into contact with the test substance with which the latter has been impregnated
- (c) The manner in which the inoculum is placed on the surface of the medium affects its rapidity of growth. Thus, it grows much better if placed right side up than up side down or at an angle. Against these objections and in favour of the emulsion, it is easy to measure equal amounts of a standard emulsion and it spreads evenly and rapidly over the surface of the new medium so that it comes into immediate and direct contact with it and its contained chemical substance

Dilution — Beginning with 1/1,000 the dilutions were each time increased by 1,000 until 1/20,000 was reached. Between 1/20,000 and 1/100,000 the dilution was increased by arithmetical progression by 1,000, after each increase to 20,000 higher. Thus, the increases between 1/20,000 and 1/40,000 was by steps of 2,000 and between 1/40,000 and 1/60,000 by steps of 3,000, and so on. Between 1/100,000 and 1/500,000 the increase was performed in the same manner but by steps of 10,000 for each increase of 100,000. The steps in dilution between 1/1,000,000 and 1/2,000,000 were 100,000.

All the dilutions indicated by the above system were utilized in each series of tests up to the maximum shown in Tables I to VI (*Appendix*), but when all the tests were negative for any dilution this was omitted from the tables concerned as otherwise they would have been made unwieldy by the inclusion of many columns showing only negative results. It should also be noted that where any test ends in a negative sign it means that for that particular substance and organism this was the highest dilution tested.

The tables have been arranged in exactly the same way as in our previous paper so that comparison is easy.

REFERENCE

MAPLESTONE, P. A., and DEY, N. C. *Ind Jour Med Res*, **25**, p. 603 (1938)

APPENDIX

TABLE I

Fungistatic tests

Dilution —	<i>Epidermophyton floccosum</i>														
	1/1,000	1/2,000	1/4,000	1/5 000	1/8,000	1/11 000	1/13,000	1/14,000	1/20,000	1/22,000	1/43,000	1/72,000	1/76,000	1/95,000	1/220,000
Quinine bihydrochlor	+														
Iodine	-	+													
Fuchsin (basic)	-	+													
Paranitrophenol (pure)	-	-	+												
Acid benzoic	-	-		+											
Acid salicylic	-	-	-	+											
Mercurochrome	-	-	-	+											
Hydrarg perchlor ⁸	-	-	-	+											
Gentian violet	-	-	-	-	+										
Clove oil	-	-	-	-	-	+									
Thymol	-	-	-	-	-	-	+								
Abracide	-	-	-	-	-	-	+								
Crystal violet	-	-	-	-	-	-	-	+							
Acridavine	-	-	-	-	-	-	-	-	+						
Cinnamon oil	-	-	-	-	-	-	-	-	-	+					
Brilliant green	-	-	-	-	-	-	-	-	-	-	+				
Brilliant green	}	-	-	-	-	-	-	-	-	-	-	+			
Gentian violet		-	-	-	-	-	-	-	-	-	-	-	-	+	
Malachite green		-	-	-	-	-	-	-	-	-	-	-	+		
Brilliant green	}	-	-	-	-	-	-	-	-	-	-	-	-	+	
Crystal violet		-	-	-	-	-	-	-	-	-	-	-	-	-	+
Merfend		-	-	-	-	-	-	-	-	-	-	-	-	-	+

+ = Growth -

- = No growth

TABLE I—*contd*

Dilution —	<i>Achorion violaceum</i>												
	1/1,000	1/2,000	1/4,000	1/5,000	1/6,000	1/12,000	1/20,000	1/49,000	1/64,000	1/120,000	1/150,000	1/220,000	1/330,000
Quinine bihydrochlor.	+												
Iodine .	-	-	+										
Fuchsin (basic)	-	+											
Paranitrophenol (pure)	-	-	+										
Acid benzoic	-	-	-	+									
Acid salicylic	-	-	-	+									
Mercurochrome	-	-	-	+									
Hydrarg perchlor	-	-	-	-	+								
Gentian violet	-	-	-	-	+								
Clove oil .	-	-	-	-	-	+							
Thymol	-	-	-	-	-	-	+						
Abracide	-	-	-	-	-	-	+						
Crystal violet	-	-	-	-	-	-	-	+					
Acriflavine	-	-	-	-	+								
Cinnamon oil	-	-	-	-	-	-	+						
Brilliant green	-	-	-	-	-	-	-	-	-	-	+		
Brilliant green } Gentian violet }	-	-	-	-	-	-	-	-	-	+			
Malachite green	-	-	-	-	-	-	-	-	+				
Brilliant green } Crystal violet }	-	-	-	-	-	-	-	-	-	-	-	+	
Merfenil	-	-	-	-	-	-	-	-	-	-	-	-	+

+ = Growth

- = No growth

TABLE I—*contd*

Dilution :—	<i>Microsporum audouinii</i>														
	1/1,000	1/4,000	1/6,000	1/8,000	1/10,000	1/12,000	1/20,000	1/40,000	1/60,000	1/95,000	1/150,000	1/180,000	1/190,000	1/220,000	1/280,000
Quinino bihydrochlor	+														
Iodine	-	+													
Fuchsin (basic)	-	-	-	-	-	+									
Paramitrophenol (pure)	-	+													
Acid benzoic	-	-	+												
Acid salicylic	-	-	+												
Mercurochrome	-	-	-	-	+										
Hydrarg perchlor	-	-	-	+											
Gentian violet	-	-	-	-	-	-	+								
Clove oil	-	-	-	-	-	+									
Thymol	-	-	-	-	-	-	+								
Abracide	-	-	-	-	-	-	+								
Crystal violet	-	-	-	-	-	-	-	-	+						
Acriflavine	-	-	-	-	-	-	-	-	+						
Cinnamon oil	-	-	-	-	-	-	-	+							
Brilliant green	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Brilliant green }	-	-	-	-	-	-	-	-	-	-	+				
Gentian violet }	-	-	-	-	-	-	-	-	-	-	-	+			
Malachite green	-	-	-	-	-	-	-	-	-	-	-	+			
Brilliant green }	-	-	-	-	-	-	-	-	-	-	-	-	+		
Crystal violet }	-	-	-	-	-	-	-	-	-	-	-	-	-	+	
Merfenil	-	-	-	-	-	-	-	-	-	-	-	-	-	+	

+

= Growth

-

= No growth

+ = Growth

- = No growth

TABLE I—*concl'd*

Dilution —	<i>Achorion actoni</i>													
	1/1,000	1/4,000	1/5,000	1/6,000	1/12,000	1/15,000	1/20,000	1/80,000	1/85,000	1/160,000	1/190,000	1/280,000.	1/330,000	1/400,000
Quinine bihydrochlor	+													
Iodine	-	+												
Fuchsin (basic)	-	-	-	-	+									
Paranitrophenol (pure)	-	-	+											
Acid benzoic	-	-	+											
Acid salicylic	-	-	+											
Mercurochrome	-	-	+											
Hydrarg perchlor	-	-	-	+										
Gentian violet	-	-	-	-	-	+								
Clove oil	-	-	-	-	+									
Thymol	-	-	-	-	-	-	+							
Abracide	-	-	-	-	-	-	+							
Crystal violet	-	-	-	-	-	-	-	-	+					
Acriflavine	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Cinnamon oil	-	-	-	-	+									
Brilliant green	-	-	-	-	-	-	-	-	-	-	-	+		
Brilliant green } Gentian violet }	-	-	-	-	-	-	-	-	-	+				
Malachite green	-	-	-	-	-	-	-	-	-	-	+			
Brilliant green } Crystal violet }	-	-	-	-	-	-	-	-	-	-	-	-	+	
Merfenil	-	-	-	-	-	-	-	+						

+ = Growth

- = No growth.

TABLE II.

Bacteriostatic effect on a strain of Staphylococcus aureus isolated from a mixed infection with Achorion violaceum

Dilution —	1/1,000	1/2,000	1/5,000	1/6,000	1/8,000	1/11,000	1/19,000	1/24,000	1/38,000	1/76,000	1/90,000	1/120,000	1/150,000	1/160,000	1/2,000,000
Quinine bihydrochlor	+														
Acid benzoic	-	+													
Acid salicylic	-	-	+												
Thymol	-	-	+												
Iodine	-	-	-	+											
Clove oil	-	-	-	+											
Abracide	-	-	-	+											
Acridlavine	-	-	-	+											
Cinnamon oil	-	-	-	-	+										
Fuchsin (basic)	-	-	-	-	-	+									
Merouochrome	-	-	-	-	-	+									
Brilliant green	-	-	-	-	-	-	+								
Malachite green	-	-	-	-	-	-	-	+							
Paramitrophenol (pure)	-	-	-	-	-	-	-	-	+						
Hydrarg perchlor	-	-	-	-	-	-	-	-	-	+					
Gentian violet	-	-	-	-	-	-	-	-	-	-	+				
Gentian violet }	-	-	-	-	-	-	-	-	-	-	-	+			
Brilliant green }	-	-	-	-	-	-	-	-	-	-	-	-	+		
Crystal violet	-	-	-	-	-	-	-	-	-	-	-	-	-	+	
Brilliant green }	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Crystal violet }	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Merfenal	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+

+ = Growth

- = No growth

TABLE III.

*Fungicidal tests.**Epidermophyton floccosum* (= *cruris*)

TIME —	1 MINUTE										
Dilution —	1/1,000	1/2,000	1/3,000	1/4,000	1/5,000	1/6,000	1/11,000	1/12,000	1/26,000	1/46,000	1/49,000
Quinine bihydrochlor	+										
Paranitrophenol (pure)	+										
Acid benzoic	+										
Clove oil	+										
Cinnamon oil	+										
Fuchsin (basic)	+										
Mercurochrome	-	+									
Thymol	-	+									
Acid salicylic	-	-	+								
Abracide	-	-	+								
Gentian violet	-	-	-	+							
Acriflavine	-	-	-	-	+						
Crystal violet	-	-	-	-	+						
Malachite green	-	-	-	-	-	+					
Hydrarg perchlor	-	-	-	-	-	+					
Iodine	-	-	-	-	-	-	+				
Brilliant green	-	-	-	-	-	-	-	+			
Brilliant green	-	-	-	-	-	-	-	-	+		
Gentian violet	-	-	-	-	-	-	-	-	-	+	
Brilliant green	-	-	-	-	-	-	-	-	-	-	+
Crystal violet	-	-	-	-	-	-	-	-	-	-	-
Merfenil	-	-	-	-	-	-	-	-	-	-	+

+ = Growth

- = No growth

TABLE III—*contd*

TIME —	10 MINUTES										
Dilution —	1/1,000	1/2,000	1/3,000	1/4,000	1/5,000	1/7,000	1/11,000	1/16,000	1/40,000	1/55,000	1/180,000
Quinine bihydrochlor	+										
Parantrophanol (pure)	+										
Acid benzoic	+										
Clove oil	-	+									
Cinnamon oil	-	-	-	+							
Fuchsin (basic)	-	-	+								
Mercurchrome	-	+									
Thymol	-	-	+								
Acid salicylic	-	-	+								
Abracide	-	-	-	+							
Gentian violet	-	-	-	+							
Acridavine	-	-	-	-	+						
Crystal violet	-	-	-	-	+						
Malachite green	-	-	-	-	-	+					
Hydrarg perchlor	-	-	-	-	-	+					
Iodine	-	-	-	-	-	-	+				
Brilliant green	-	-	-	-	-	-	-	+			
Brilliant green } Gentian violet }	-	-	-	-	-	-	-	-	+		
Brilliant green } Crystal violet }	-	-	-	-	-	-	-	-	-	+	
Merfenil	-	-	-	-	-	-	-	-	-	-	+

+ = Growth

- = No growth

TABLE III—*contd.*

TIME —	30 MINUTES													
Dilution —	1/1,000	1/2,000	1/3,000	1/4,000	1/5,000	1/6,000	1/9,000	1/14,000	1/16,000	1/17,000	1/18,000	1/55,000	1/60,000	1/200,000
Quinine bihydrochlor	+													
Paranitrophenol (pure)	+													
Acid benzoic	-	+												
Clove oil	-	+												
Cinnamon oil	-	-	-	+										
Fuchsin (basic)	-	-	-	+										
Mercurochrome	-	-	+											
Thymol	-	-	-	-	+									
Acid salicylic	-	-	+											
Abracido	-	-	-	-	-	+								
Gentian violet	-	-	-	+										
Acridine	-	-	-	-	+									
Crystal violet	-	-	-	-	-	-	+							
Malachite green	-	-	-	-	-	-	-	-	-	+				
Hydrarg perchlor	-	-	-	-	-	-	-	-	-	-	+			
Iodine	-	-	-	-	-	-	-	+						
Brilliant green	-	-	-	-	-	-	-	-	+					
Brilliant green }	-	-	-	-	-	-	-	-	-	-	-	+		
Gentian violet }	-	-	-	-	-	-	-	-	-	-	-	-		
Brilliant green }	-	-	-	-	-	-	-	-	-	-	-	-	+	
Crystal violet }	-	-	-	-	-	-	-	-	-	-	-	-	-	
Merfeml	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+ = Growth.

- = No growth

TABLE III—*contd*

TIME —	1 HOUR													
Dilution —	1/1,000	1/2,000	1/3,000	1/4,000	1/6,000	1/7,000	1/8,000	1/10,000	1/14,000	1/20,000	1/24,000	1/55,000	1/80,000	1/200,000
Quinine bihydrochlor	+													
Paranitrophenol (pure)	-	+												
Acid benzoic	-	-	+											
Clove oil	-	+												
Cinnamon oil	-	-	-	+										
Fuchsin (basic)	-	-	-	-	+									
Mercurochrome	-	-	-	+										
Thymol	-	-	-	-	-	+								
Acid salicylic	-	-	+											
Abracide	-	-	-	-	-	+								
Gentian violet	-	-	-	-	-	-	+							
Acriflavine	-	-	-	-	+									
Crystal violet	-	-	-	-	-	-	-	-	+					
Malachite green	-	-	-	-	-	-	-	-	-	+				
Hydrarg perchlor	-	-	-	-	-	-	-	-	-	-	+			
Iodine	-	-	-	-	-	-	-	-	+					
Brilliant green	-	-	-	-	-	-	-	-	-	+				
Brilliant green }	-	-	-	-	-	-	-	-	-	-	-	+		
Gentian violet }	-	-	-	-	-	-	-	-	-	-	-	-	+	
Brilliant green }	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Crystal violet }	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Merfenil	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+ = Growth

- = No growth.

TABLE III—*concl'd.*

TIME —	24 HOURS								
Dilution —	1/1,000	1/5,000	1/10,000	1/15,000	1/20,000	1/24,000	1/60,000	1/100,000	1/200,000
Quinine bihydrochlor	+								
Paranitrophenol (pure)	-	+							
Acid benzoic	-	+							
Clove oil	-	+							
Cinnamon oil	-	-							
Fuchsin (basic)	-	-	-						
Mercurochrome	-	+							
Thymol	-	-	-						
Acid salicylic	-	+							
Abracide	-	-	-						
Gentian violet	-	-	-						
Acriflavine	-	-	-	-					
Crystal violet	-	-	-	-	-				
Malachite green	-	-	-	-	-				
Hydrarg perchlor	-	-	-	-	-	-			
Iodine	-	-	-	-	+				
Brilliant green	-	-	-	-	+				
Brilliant green } Gentian violet }	-	-	-	-	-	-	-		
Brilliant green } Crystal violet }	-	-	-	-	-	-	-	-	
Merfenil	-	-	-	-	-	-	-	-	-

+ = Growth

- = No growth

TABLE IV

*Fungicidal tests**Achorion violaceum*

TIME —	1 MINUTE									
Dilution —	1/1,000	1/2,000	1/3,000	1/4,000	1/5,000	1/6,000	1/7,000	1/10,000	1/11,000	1/12,000
Quinine bihydrochlor	+									
Paranitrophenol (pure)	+									
Clove oil	+									
Acid benzoic	+									
Malachite green	+									
Acridlavine	+									
Mercurochrome	+									
Fuchsin (basic)	+									
Cinnamon oil	+									
Acid salicylic	—	+								
Brilliant green	—	—	+							
Crystal violet	—	—	+							
Brilliant green	—	—	+							
Gentian violet	—	—	—	+						
Gentian violet	—	—	—	+						
Hydrarg perchlor	—	—	—	+						
Crystal violet	—	—	—	—	+					
Thymol	—	—	—	—	—	+				
Abracide	—	—	—	—	—	—	+			
Merfenol	—	—	—	—	—	—	—	+		
Brilliant green	—	—	—	—	—	—	—	—	+	
Iodine	—	—	—	—	—	—	—	—	—	+

+ = Growth

— = No growth

TABLE IV—*contd*

TIME —	10 MINUTES									
Dilution —	1/1,000	1/2,000	1/3,000	1/4,000	1/5,000	1/6,000	1/7,000	1/15,000	1/16,000	1/40,000
Quinine bihydrochlor	+									
Paramitrophenol (pure)	+									
Clove oil	+									
Acid benzoic	+									
Malachite green	+									
Acriflavine	+									
Mercurochrome	+									
Fuchsin (basic)	—	+								
Cinnamon oil	—	+								
Acid salicylic	—	—	+							
Brilliant green	—	—	±							
Crystal violet										
Brilliant green	—	—	—	+						
Gentian violet				+						
Gentian violet	—	—	—	+						
Hydrarg perchlor	—	—	—	—	+					
Crystal violet	—	—	—	—	—	—	+			
Thymol	—	—	—	—	—	+				
Abracide	—	—	—	—	—	—	+			
Merfenil	—	—	—	—	—	—	—	—	—	+
Brilliant green	—	—	—	—	—	—	—	—	+	
Iodine	—	—	—	—	—	—	—	+		

+ = Growth — = No growth

TABLE IV—*contd*

TIME —	30 MINUTES										
Dilution —	1/1,000	1/2,000	1/3,000	1/4,000	1/5,000	1/7,000	1/8,000	1/9,000	1/15,000	1/16,000	1/30,000
Quinine bihydrochlor	+										
Paranitrophenol (pure)	+										
Clove oil	+										
Acid benzoic	+										
Malachite green	-										
Acridavine	+										
Mercurochrome	+										
Fuchsin (basic)	-	+									
Cinnamon oil	-	-	+								
Acid salicylic	-	-	+								
Brilliant green	-	-	+								
Crystal violet	-	-	+								
Brilliant green	-	-	-	+							
Gentian violet	-	-	-	+							
Gentian violet	-	-	-	+							
Hydrarg perchlor	-	-	-	-	+						
Crystal violet	-	-	-	-	-	-	-	+			
Thymol	-	-	-	-	-	+					
Abracide	-	-	-	-	-	-	+				
Merfenil	-	-	-	-	-	-	-	-	-	-	+
Brilliant green	-	-	-	-	-	-	-	-	-	+	
Iodine	-	-	-	-	-	-	-	-	+		

+ = Growth

- = No growth

TABLE IV—*contd*

TIME —	1 HOUR										
Dilution —	1/1,000	1/2,000	1/3,000	1/5,000	1/6,000	1/7,000	1/8,000	1/13,000	1/15,000	1/16,000	1/34,000.
Quinine bihydrochlor	+										
Parantrophanol (pure)	+										
Clove oil	—	+									
Acid benzoic	+										
Malachite green	—	—	+								
Acriflavine	+										
Mercurochrome	+										
Fuchsin (basic)	—	—	—	—	+						
Cinnamon oil	—	—	—	+							
Acid salicylic	—	—	+								
Brilliant green	—	—	—	—	+						
Crystal violet	—	—	—	—	+						
Brilliant green	—	—	—	—	+						
Gentian violet	—	—	—	—	—	+					
Gentian violet	—	—	—	—	—	+					
Hydrarg perchlor	—	—	—	—	—	+					
Crystal violet	—	—	—	—	—	—	—	+			
Thymol	—	—	—	—	—	+					
Abracide	—	—	—	—	—	—	+				
Merfemil	—	—	—	—	—	—	—	—	—	—	+
Brilliant green	—	—	—	—	—	—	—	—	—	+	
Iodine	—	—	—	—	—	—	—	—	+		

+ = Growth

— = No growth

TABLE IV—*conold*

Time —	24 Hours						
Dilution —	1/1,000	1/3,000	1/4,000	1/5,000	1/10,000	1/20,000	1/100,000
Quinine bihydrochlor	+						
Paranitrophenol (pure)	-	+					
Clove oil	-	-	+				
Acid benzoic	-	-	-	+			
Malachite green	-	-	-	-			
Acridavine	-	-	-	-			
Mercurochrome	-	-	-	-			
Fuchsin (basic)	-	-	-	-			
Cinnamon oil	-	-	-	+			
Acid salicylic	-	-	-	+			
Brilliant green }	-	-	-	-	-		
Crystal violet }							
Brilliant green }	-	-	-	-	-		
Gentian violet }							
Gentian violet	-	-	-	-	+		
Hydrarg perchlor	-	-	-	-	-		
Crystal violet	-	-	-	-	-	-	
Thymol	-	-	-	-	-		
Abracide	-	-	-	-	-		
Merfenu	-	-	-	-	-	-	-
Brilliant green	-	-	-	-	-	+	
Iodine	-	-	-	-	-	+	

+ = Growth

- = No growth

TABLE V

*Fungicidal tests**Microsporium audouinii*

TIME —	1 MINUTE										
Dilution —	1/1,000	1/2,000	1/3,000	1/4,000	1/7,000	1/9,000	1/10,000	1/11,000	1/17,000	1/28,000	1/90,000
Quinine bihydrochlor	+										
Paranitrophenol (pure)	+										
Acid benzoic	+										
Mercurochrome	+										
Acid salicylic	-	+									
Fuchsin (basic)	-	+									
Clove oil	-	-	+								
Cinnamon oil	-	-	+								
Acridavine	-	-	-	+							
Thymol	-	-	-	-	+						
Abraide	-	-	-	-	+						
Gentian violet	-	-	-	-	+						
Hydrarg perchlor	-	-	-	-	-	+					
Crystal violet	-	-	-	-	-	-	+				
Iodine	-	-	-	-	-	-	-	+			
Brilliant green	-	-	-	-	-	-	-	+			
Malachite green	-	-	-	-	-	-	-	+			
Brilliant green } Gentian violet }	-	-	-	-	-	-	-	-	+		
Brilliant green } Crystal violet }	-	-	-	-	-	-	-	-	-	+	
Merfenil	-	-	-	-	-	-	-	-	-	-	+

+ = Growth

- = No growth

TABLE V—*contd*

TIME —	10 MINUTES														
Dilution —	1/1,000	1/2,000	1/3,000	1/4,000	1/5,000	1/7,000	1/8,000	1/9,000	1/10,000	1/14,000	1/18,000	1/22,000	1/40,000	1/49,000	1/200,000
Quinine bihydrochlor	+														
Paranutrophenol (pure)	+														
Acid benzoic	-	+													
Mercurochrome	-	-	+												
Acid salicylic	-	-	+												
Fuchsin (basic)	-	-	-	-	+										
Clove oil	-	-	-	-	+										
Cinnamon oil	-	-	-	-	+										
Acridavine	-	-	-	+											
Thymol	-	-	-	-	-	+									
Abracide	-	-	-	-	-	+									
Gentian violet	-	-	-	-	-	-	+								
Hydrarg perchlor	-	-	-	-	-	-	-	+							
Crystal violet	-	-	-	-	-	-	-	-	+						
Iodine	-	-	-	-	-	-	-	-	-	+					
Brilliant green	-	-	-	-	-	-	-	-	-	-	+				
Malachite green	-	-	-	-	-	-	-	-	-	-	-	+			
Brilliant green	-	-	-	-	-	-	-	-	-	-	-	-	+		
Gentian violet	-	-	-	-	-	-	-	-	-	-	-	-	-	+	
Brilliant green	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Crystal violet	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Merfenil	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+

+ = Growth

- = No growth

TABLE V—*contd*

TIME —	30 MINUTES														
Dilution —	1/1,000	1/2,000	1/3,000	1/4,000	1/6,000	1/8,000	1/9,000	1/12,000	1/15,000	1/20,000	1/24,000	1/28,000	1/43,000	1/49,000	1/200,000
Quinine bihydrochlor	+														
Paranitrophenol (pure)	+														
Acid benzoic	—	+													
Mercurochrome	—	—	+												
Acid salicylic	—	—	+												
Fuchsin (basic)	—	—	—	—	+										
Clove oil	—	—	—	—	+										
Cinnamon oil	—	—	—	—	+										
Acridlavine	—	—	—	+											
Thymol	—	—	—	—	—	+									
Abracide	—	—	—	—	—	+									
Gentian violet	—	—	—	—	—	—	—	—	+						
Hydrarg perchlor	—	—	—	—	—	—	+								
Crystal violet	—	—	—	—	—	—	—	—	—	—	+				
Iodine	—	—	—	—	—	—	—	+							
Brilliant green	—	—	—	—	—	—	—	—	—	+					
Malachite green	—	—	—	—	—	—	—	—	—	—	—	+			
Brilliant green }	—	—	—	—	—	—	—	—	—	—	—	—	+		
Gentian violet }															
Brilliant green }	—	—	—	—	—	—	—	—	—	—	—	—	—	+	
Crystal violet }															
Merfenil	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

+ = Growth

- = No growth

TABLE V—contd

TIME —	1 HOUR													
Dilution —	1/1,000	1/2,000	1/3,000	1/4,000	1/6,000	1/9,000	1/10,000	1/12,000	1/20,000	1/24,000	1/30,000	1/60,000	1/90,000	1/200,000
Quinine bihydrochlor	+													
Paramitrophenol (pure)	+													
Acid benzoic	-	+												
Mercurochrome	-	-	+											
Acid salicylic	-	-	+											
Fuchsin (basic)	-	-	-	-		+								
Clove oil	-	-	-	-	+									
Cinnamon oil	-	-	-	-	+									
Acridavine	-	-	-	+										
Thymol	-	-	-	-	-	+								
Abracide	-	-	-	-	-	-	+							
Gentian violet	-	-	-	-	-	-	-	-	+					
Hydrarg perchlor	-	-	-	-	-	+								
Crystal violet	-	-	-	-	-	-	-	-	-	-	+			
Iodine	-	-	-	-	-	-	-	+						
Brilliant green	-	-	-	-	-	-	-	-	-	+				
Malachite green	-	-	-	-	-	-	-	-	-	-	+			
Brilliant green } Gentian violet }	-	-	-	-	-	-	-	-	-	-	-	+		
Brilliant green } Crystal violet }	-	-	-	-	-	-	-	-	-	-	-	-	+	
Merfenil	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+ = Growth

- = No growth

TABLE V—*concl'd.*

TIME —	24 HOURS										
Dilution —	1/1,000	1/3,000	1/5,000	1/6,000	1/9,000	1/10,000	1/20,000	1/30,000	1/60,000	1/100,000	1/200,000
Quinine bihydrochlor	+										
Paranitrophenol (pure)	-	-	-								
Acid benzoic	-	+									
Mercurochrome	-	-	-	-	-	-					
Acid salicylic	-	+									
Fuchsin (basic)	-	-	-	-	-	-					
Clove oil	-	-	-	+							
Cinnamon oil	-	-	-	-	+						
Acriflavine	-	-	-	-	-	-					
Thymol	-	-	-	-	-	-					
Abracide	-	-	-	-	-	-					
Gentian violet	-	-	-	-	-	-	-				
Hydrarg perchlor	-	-	-	-	-	-	-				
Crystal violet	-	-	-	-	-	-	-	-			
Iodine	-	-	-	-	-	-	-	-			
Brilliant green	-	-	-	-	-	-	-	-			
Malachite green	-	-	-	-	-	-	-	-			
Brilliant green	-	-	-	-	-	-	-	-	-		
Gentian violet	-	-	-	-	-	-	-	-	-		
Brilliant green	-	-	-	-	-	-	-	-	-	-	
Crystal violet	-	-	-	-	-	-	-	-	-	-	
Merfenil	-	-	-	-	-	-	-	-	-	-	-

+ = Growth

- = No growth

TABLE VI

*Fungicidal tests**Achorion actoni*

TIME —	1 MINUTE									
Dilution —	1/1,000	1/3,000	1/4,000	1/5,000	1/8,000	1/10,000	1/13,000	1/19,000	1/28,000	1/49,000
Quinine bishydrochlor	+									
Paramitrophenol (pure)	+									
Acid benzoic	+									
Acid salicylic	+									
Mercurochrome	+									
Fuchsin (basic)	+									
Acridavine	+									
Clove oil	—	+								
Thymol	—	+								
Cinnamon oil	—	+								
Abracide	—	—	+							
Gentian violet	—	—	+							
Hydrarg perchlor	—	—	—	+						
Crystal violet	—	—	—	—	+					
Iodine	—	—	—	—	—	+				
Malachite green	—	—	—	—	—	+				
Brilliant green	—	—	—	—	—	—	+			
Brilliant green } Gentian violet }	—	—	—	—	—	—	—	+		
Brilliant green } Crystal violet }	—	—	—	—	—	—	—	—	+	
Merfennil	—	—	—	—	—	—	—	—	—	+

+ = Growth

— = No growth

TABLE VI—*contd*

TIME —	10 MINUTES									
Dilution —	1/1,000	1/2,000	1/3,000	1/4,000	1/5,000	1/10,000	1/15,000	1/20,000	1/30,000	1/95,000
Quinine bihydrochlor	+									
Paranitrophenol (pure)	+									
Acid benzoic	+									
Acid salicylic	+									
Mercurochrome	+									
Fuchsin (basic)	+									
Acridlavine	-	+								
Clove oil	-	-	+							
Thymol	-	-	+							
Cinnamon oil	-	-	-	+						
Abracide	-	-	-	+						
Gentian violet	-	-	-	+						
Hydrarg perchlor	-	-	-	-	+					
Crystal violet	-	-	-	-	-	+				
Iodine	-	-	-	-	-	+				
Malachite green	-	-	-	-	-	+				
Brilliant green	-	-	-	-	-	-	+			
Brilliant green	-	-	-	-	-	-	-	+		
Gentian violet	-	-	-	-	-	-	-	-		
Brilliant green	-	-	-	-	-	-	-	-	+	
Crystal violet	-	-	-	-	-	-	-	-	-	
Merfanil	-	-	-	-	-	-	-	-	-	+

+ = Growth

- = No growth

TABLE VI—*contd*

TIME —	30 MINUTES											
Dilution —	1/1,000	1/2,000	1/3,000	1/4,000	1/5,000	1/6,000	1/7,000	1/10,000	1/15,000	1/24,000	1/38,000	1/200,000
Quinine bihydrochlor	+											
Paranitrophenol (pure)	+											
Acid benzoic	+											
Acid salicylic	+											
Mercurochrome	+											
Fuchsin (basic)	-	+										
Acriflavine	-	-	-	-	+							
Clove oil	-	-	+									
Thymol	-	-	-	-	-	+						
Cinnamon oil	-	-	-	+								
Abracide	-	-	-	-	-	-	+					
Gentian violet	-	-	-	-	+							
Hydrarg perchlor	-	-	-	-	-	+						
Crystal violet	-	-	-	-	-	-	-	-	+			
Iodine	-	-	-	-	-	-	-	+				
Malachite green	-	-	-	-	-	-	-	+				
Brilliant green	-	-	-	-	-	-	-	-	+			
Brilliant green }	-	-	-	-	-	-	-	-	-	+		
Gentian violet }	-	-	-	-	-	-	-	-	-	-	+	
Brilliant green }	-	-	-	-	-	-	-	-	-	-	-	+
Crystal violet }	-	-	-	-	-	-	-	-	-	-	-	-
Merfenil	-	-	-	-	-	-	-	-	-	-	-	+

+ = Growth

- = No growth

TABLE VI—*contd*

TIME —	1 HOUR											
Dilution —	1/1,000	1/2,000	1/3,000	1/4,000	1/5,000	1/6,000	1/10,000	1/12,000	1/16,000	1/30,000	1/46,000	1/200,000
Quinine bihydrochlor	+											
Paranitrophenol (pure)	+											
Acid benzoic	-	+										
Acid salicylic	-	+										
Mercurochrome	-	+										
Fuchsin (basic)	-	-	-	+								
Acridflavine	-	-	-	-	+							
Clove oil	-	-	+									
Thymol	-	-	-	-	-	+						
Cinnamon oil	-	-	-	+								
Abracide	-	-	-	-	-	+						
Gentian violet	-	-	-	-	+							
Hydrarg perchlor	-	-	-	-	-	+						
Crystal violet	-	-	-	-	-	-	-	-	-	+		
Iodine	-	-	-	-	-	-	+					
Malachite green	-	-	-	-	-	-	-	+				
Brilliant green	-	-	-	-	-	-	-	-	+			
Brilliant green } Gentian violet }	-	-	-	-	-	-	-	-	-	+		
Brilliant green } Crystal violet }	-	-	-	-	-	-	-	-	-	-	+	
Merfanil	-	-	-	-	-	-	-	-	-	-	-	-

+ = Growth

- = No growth.

TABLE VI—*concl'd*

TIME —	24 Hours												
Dilution —	1/1,000	1/2,000	1/3,000	1/5,000	1/8,000	1/8,000	1/10,000	1/15,000	1/16,000	1/20,000	1/30,000	1/100,000	1/200,000
Quinine bihydrochlor	+												
Parantrophanol (pnre)	-	-	+										
Acid benzoic	-	+											
Acid salicylic	-	-	+										
Mercurochrome	-	-	-	-									
Fuchsin (basic)	-	-	-	-									
Acridavine	-	-	-	-	+								
Clove oil	-	-	-	-	-								
Thymol	-	-	-	-	-	+							
Cinnamon oil	-	-	-	-	-	-	+						
Abracide	-	-	-	-	-	+							
Gentian violet	-	-	-	-	-	-	-						
Hydrarg perchlor	-	-	-	-	-	-	-						
Crystal violet	-	-	-	-	-	-	-	-	-	-	-		
Iodine	-	-	-	-	-	-	-	-	+				
Malachite green	-	-	-	-	-	-	-	+					
Brilliant green	-	-	-	-	-	-	-	-	-	+			
Brilliant green	}	-	-	-	-	-	-	-	-	-	-	-	
Gentian violet		-	-	-	-	-	-	-	-	-	-	-	
Brilliant green	}	-	-	-	-	-	-	-	-	-	-	-	
Crystal violet		-	-	-	-	-	-	-	-	-	-	-	
Merfennl	-	-	-	-	-	-	-	-	-	-	-	-	-

+ = Growth

- = No growth

RAT-FLEAS OF CALCUTTA INVESTIGATED FROM A POINT OF VIEW OF EPIDEMIOLOGY OF PLAGUE

BY

S RAGHVENDER RAO

(*From the Plague Department, Hyderabad-Deccan*)

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ONE of the notable features of plague epidemics in India has been the variation in the severity of the incidence in different parts of the country having apparently the same environmental conditions. In certain cities such as Bombay, Calcutta and Madras this variation has been very distinct. Madras has been almost free from plague from the very beginning and the causes for this have been the subject of a detailed study by the British Plague Research Commission (1912), and later by King, George, Mankikar and Jesudasan (1930*a*, 1930*b*), and King and Pandit (1931) as far as the rat and flea population of the city is concerned.

Calcutta, while having a population slightly larger than that of Bombay, suffered from plague to a much less extent and the disease, at least in its epidemic form, disappeared from the city at a much earlier date (Table I). Again, in Calcutta, though the infection was introduced earlier than in Bombay, it took some time for the disease to take an epidemic form. Even then the incidence of plague in these Calcutta epidemics was not so severe as compared to that experienced in some other cities in India. In Bombay, on the other hand, severe epidemics of plague began to appear shortly after the introduction of infection and it was only by the end of the year 1923 that severe plague epidemics were not regular annual features in that city.

After the differentiation of the three important species of rat-fleas in India by Rothschild (1914), and the finding of Hirst (1913) that the newly discovered species *Xenopsylla astia* was the common rat-flea of Colombo and also of another plague-free city in India, viz. Madras, much attention was paid to this rat-flea species factor in determining the incidence of plague in any place. This view was further strengthened by the experiments of Philip and Hirst (1917) who put forward the hypothesis that the relative freedom from plague of Ceylon and certain parts of South India was to be attributed to the relative inefficiency of *Xenopsylla astia*

TABLE I.

Plague mortality in Bombay and Calcutta

Year	BOMBAY		CALCUTTA	
	Plague deaths	Mortality rate per 100,000	Plague deaths	Mortality rate per 100,000
1895			10	0 8
1896	1,936	24 2		
1897	11,003	1385 2	11	0 8
1898	16,821	2130 0	239	26 5
1899	15,860	2020 0	2,029	220 7
1900	13,285	1701 2	8,275	883 0
1901	18,694	2409 0	8,010	841 1
1902	13,786	1731 1	7,538	756 8
1903	20,751	2540 8	8,224	846 9
1904	13,504	1613 3	4,703	479 6
1905	14,171	1652 8	7,272	734 6
1906	10,802	1230 7	2,607	260 9
1907	6,379	710 3	3,589	355 8
1908	5,348	582 3	1,781	174 9
1909	5,186	552 4	2,117	206 0
1910	3,641	379 6	1,262	121 7
1911	3,997	408 1	1,736	166 2
1912	1,714	171 6	1,831	174 7
1913	2,605	255 7	852	81 0
1914	2,935	282 6	442	41 9
1915	698	66 0	191	18 1
1916	1,982	183 9	78	7 3
1917	1,698	154 7	81	7 6

TABLE I—*concl.*

Year	BOMBAY		CALCUTTA.	
	Plague deaths	Mortality rate per 100,000	Plague deaths	Mortality rate per 100,000
1918	1,133	101.4	210	19.7
1919	697	61.3	834	31.2
1920	281	24.3	53	4.9
1921	801	68.1	37	3.4
1922	629	53.6	144	13.2
1923	1,329	113.3	77	7.0
1924	409	34.0	33	3.0
1925	174	14.9	9	0.8
1926	56	4.8		
1927	207	17.7		
1928	257	22.0		
1929	29	2.5		
1930	20	1.7		
1931	24	2.1		
1932	44	3.8		
1933	48	4.1		
1934	31	2.7		

as a vector of plague both from rat to rat as well as from rat to man. Similarly, Cragg (1921, 1923) examined several thousands of fleas from various places in India and showed that places with a predominating *Cheopsis* population were those that suffered most from plague, while Taylor and Chitre (1923) showed that though large tracts of land with *X. astia* as the chief rat-flea are free from plague, yet there are certain exceptions to this. Goyle (1928) has also shown that at least in the United Provinces, the predominance of *X. astia* and fairly severe incidence of plague in a place are not incompatible. To him it appeared that proximity to infected places and facilities for communication with them were more important than the flea-species factor in determining the incidence of plague.

Strickland and Roy (1930) carried out a rat-flea survey of two selected localities of Calcutta city and found that the general flea index was 0.6 with *X. astia* and *X. cheopis* indices of 0.43 and 0.16 respectively. With these results they came to the conclusion that the predominant flea in Calcutta was *X. astia* and that the absence of severe plague epidemics in the city was due to this factor.

As the above survey was on a very limited scale and the methods adopted were not free from defects* and as a preliminary to the investigation proposed to be carried out in Calcutta to find out the factors responsible for the 'long-term periodicity of plague', a more extensive rat-flea survey of the city was undertaken by the present author.

PRESENT RAT-FLEA SURVEY OF CALCUTTA

As it was not possible to trap rats regularly from all the 32 wards of the city for a long period, 10 wards were selected in such a manner as to be a complete representative sample of all the environmental and topographical conditions of the city, considered important from the point of view of the epidemiology of plague.

The following criteria formed the basis for the selection of these 10 wards —

	Wards
Wards selected for the high incidence of plague (1898-1925)	5 and 7
Wards selected for the low incidence of plague (1898-1925)	16 and 17
Wards selected for their high density of population	8 and 11
Wards selected for their low density of population	17 and 18
Wards with mostly indigenous population	1 and 11
Wards with mostly foreign population	7 and 16
Wards with business quarters and grain godowns, etc	5 and 6
Wards with mostly residential quarters	11 and 17
Sewered wards	All the above wards
Unsewered wards	18
Port area (docks, etc)	25

Table II gives the area, density of population and plague history of these wards of Calcutta city selected for the survey.

* In a personal conversation, I was informed by Dr D N Roy that they had intended to bring in all the rats from the place of collection to the laboratory in traps fully covered with bags. But as the men responsible for the trapping and collection of rats were not under their control, this rule was not at all adhered to and the rats were invariably brought in open traps exposed to the sun all the way. This factor was in all probability responsible for the low flea indices obtained by the authors.

The methods adopted for the survey work were the same as those described by Webster and Chitre (1930) Fleas collected from the rats were cleared by the usual methods and mounted for identification

TABLE II

Density of population and plague history of the 10 wards in Calcutta surveyed during the period 1936-1938

Ward number	Population	Area in acres	Density per acre	Plague mortality rate per 10,000 (1898-1925)
5 Jorabagan	39,355	243	162	64.74
7 Burra Bazar	18,690	217	86	49.87
8 Colootola	48,998	224	219	41.68
6 Jorasanko	46,633	262	176	27.45
1 Shampukur	66,633	409	163	24.59
11 Paddapukur	35,323	166	313	19.06
25 Watgunj, etc	32,463	837	39	19.06
16 Park Street	5,438	153	36	9.33
17 Victoria Terrace	2,679	128	21	7.32
18 Tangra*	11,772	953	12	*

* This ward was added only in the year 1924 to Calcutta Municipality. Consequently plague figures are not available.

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Wards with mostly residential quarters	11 and 17
Sewered wards	All the above wards
Unsewered wards	18
Port area (docks, etc.)	25

Table II gives the area, density of population and plague history of these wards of Calcutta city selected for the survey.

* In a personal conversation, I was informed by Dr. D. N. Roy that they had intended to bring in all the rats from the place of collection to the laboratory in traps fully covered with bags. But as the men responsible for the trapping and collection of rats were not under their control, this rule was not at all adhered to and the rats were invariably brought in open traps exposed to the sun all the way. This factor was in all probability responsible for the low flea indices obtained by the authors.

Besides showing this seasonal variation, the flea index figures also showed marked variation from ward to ward

TABLE IV

General flea index in different wards of Calcutta surveyed during the present investigation

Ward number *	Flea index
5 Jorabagan	4.4
7 Burra Bazar	6.5
8 Colootola	7.1
6 Jorasanko	4.6
1 Shampukur	5.7
11 Paddapukur	4.4
25 Watgunj and Hastings	6.2
16 Park Street	2.5
17 Victoria Terrace	8.7
18 Tangra	3.5

* Wards have been arranged in this table in descending order according to the incidence of plague in them in the past

The general flea index not only varied from ward to ward as seen in the above table, but also varied according to the species of rodents from which the fleas were collected. Of the three species of rats present in Calcutta *Rattus norvegicus* showed the highest flea index (9.0), while *Rattus rattus* and *Gunomys varius* showed a general flea index of 4.3 and 4.9 respectively. *Mus musculus* (house mice) showed the lowest infestation with fleas. *Crocidura coerulea* (musk rat), though not a rat, showed the same species of fleas as the other rats of the locality. The flea index for this shrew was 3.4 (Table V).

Like the general flea index, both the *cheopis* and the *astia* indices also are the highest for *Rattus norvegicus*, but the ratio of *cheopis* to *astia* is higher in *Rattus rattus*. *Gunomys varius* carried more *astia* than *cheopis*, while in the house mice (*Mus musculus*) the index is the same for both *astia* and *cheopis*. In the few bandicoots examined the *astia* index was found to be higher than the *cheopis* index.

Each species of rodents will now be considered separately with regard to their general and specific flea indices.

TABLE V

General and specific flea indices for the different species of rodents found in Calcutta

Species of rodents	Rodents examined	FLEAS FOUND			FLEA INDICES		
		X <i>astia</i>	X <i>cheopis</i>	Total	<i>Astia</i> index	<i>Cheopis</i> index	General flea index
<i>Rattus rattus</i>	220	323	618	941	1.5	2.8	4.3
<i>Rattus norvegicus</i>	264	1,604	867	2,471	5.9	3.1	9.0
<i>Gunomys varus</i>	233	859	280	1,145	3.7	1.2	4.9
<i>Mus musculus</i>	89	49	44	93	0.5	0.5	1.0
<i>Bandicoota indica</i>	7	23	5	28	3.3	0.7	4.0
<i>Crocidura coerulea</i>	62	105	103	208	1.7	1.7	3.4
TOTAL	885	2,963	1,923	4,886	3.3	2.2	5.5

Note—Though among the rats examined for fleas *Rattus rattus*, *Rattus norvegicus* and *Gunomys varus* appeared to be in almost equal numbers, this is not the actual ratio of prevalence of rats of different species in Calcutta. During this survey lasting for 12 months several thousands of rats of different species were caught and dissected. The subject-matter of the different species of rodents and their variation in number at different periods will be discussed fully in a subsequent communication.

FLEA INDICES FOR *Rattus rattus*

In this species the *cheopis* index was higher than the *astia* index in all the wards with the exception of those where the rat-fleas belonged exclusively to the species *X astia*. Such an example was ward 17. A high *cheopis* index was noted in wards 5, 6, 7, 8 and 25. A single *Rattus rattus* examined from ward 25 had all *cheopis* fleas while 6 *Rattus rattus* examined from ward 17 had all *astia* and no *cheopis*. All the wards surveyed taken together, *Rattus rattus* had a *cheopis* index of 2.8 and *astia* index of 1.5, making a general flea index of 4.3.

TABLE VI

General and specific flea indices for Rattus rattus in different wards of Calcutta surveyed during the period 1936-1938

Ward number	Rats examined	Fleas found	General flea index	SPECIFIC FLEA INDICES	
				<i>Astia</i> index	<i>Cheopis</i> index
5	53	258	4.9	1.7	3.2
7	31	182	5.9	1.1	4.8
8	11	45	4.1	0.9	3.2
6	47	210	4.4	2.1	2.3
1	34	122	3.6	1.8	1.8
11	11	31	2.8	0.9	1.9
25	1	6	6.0	0.0	6.0
16					
17	6	12	2.0	0.0	2.0
18	26	75	2.9	0.3	2.6
TOTAL	220	941	4.3	1.5	2.8

With regard to seasonal variation in flea indices for *Rattus rattus* (Table VII) from January to June there was not much difference in the *cheopis* index, but with the commencement of the monsoon the *cheopis* index fell and remained low till almost the end of December. On the other hand the *astia* index actually increased with the commencement of the winter season (see Graph). Whether the climatic factors at this period of the year become unfavourable for *cheopis* and favourable for *astia* is difficult to say. However as shown elsewhere, there is no significant difference between the relative humidity and saturation deficiency figures throughout the year and the only climatic factor that varies is the mean temperature. This during the period from October to February is much lower than during the other months of the year.

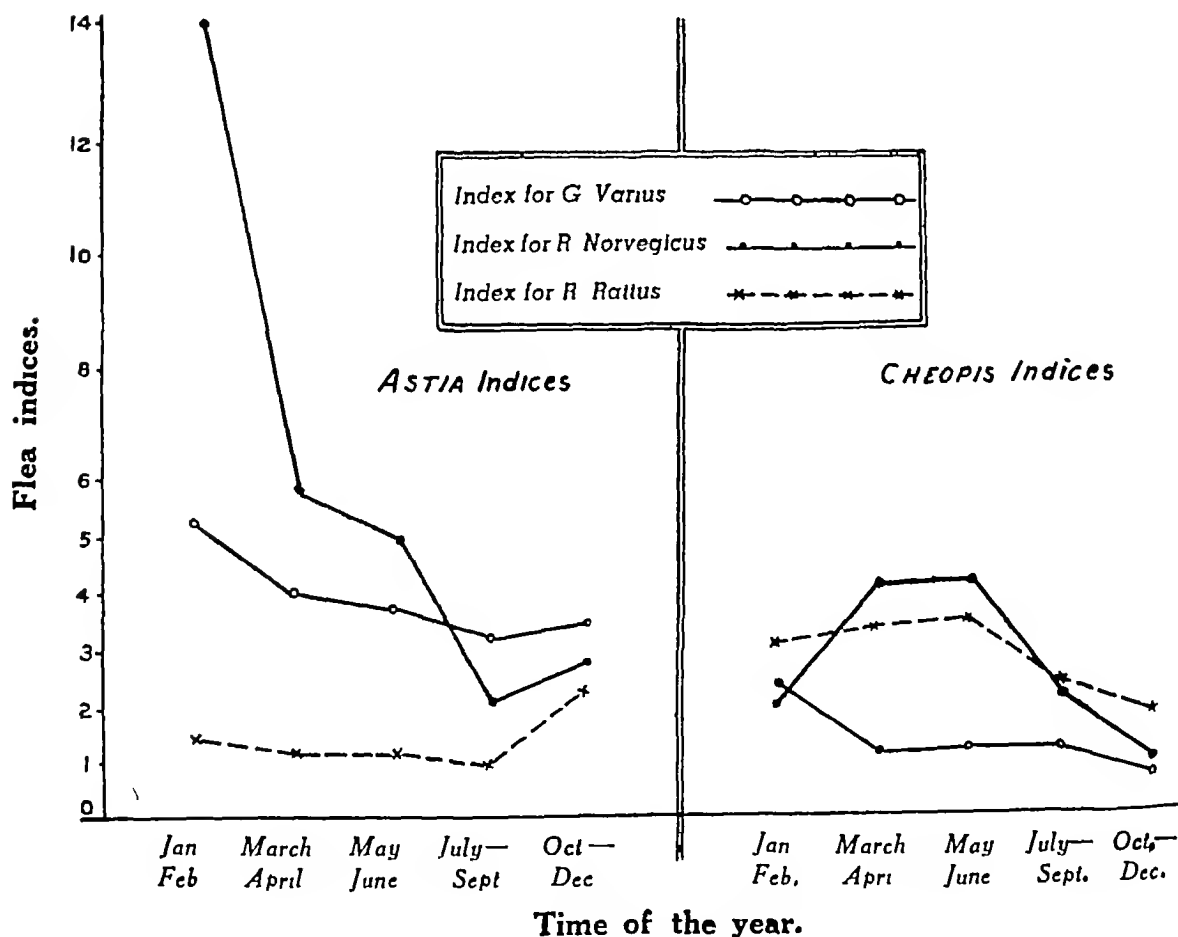
TABLE VII

Seasonal variation in general and specific flea indices for *Rattus rattus* in Calcutta

Period of examination	Number of <i>R. rattus</i> examined	<i>Astia</i> index	<i>Cheopsis</i> index
January-February 1936	30	1.4	3.1
March-April 1936	58	1.2	3.4
May-June 1936	48	1.2	3.5
July-September 1936	42	1.0	2.3
October-December 1936	42	2.3	1.8
TOTAL	220	1.5	2.8

GRAPH

Seasonal variation of specific flea indices for different species of rats found in Calcutta



FLEA INDICES FOR *Rattus norvegicus*

In this species in contrast to *Rattus rattus*, the *astia* index was higher than the *cheopsis* index in 6 out of the 10 wards surveyed (Table VIII). Individually *R. norvegicus* carried a much larger number of fleas than any other species of rodents in Calcutta. The highest flea index (12.7) for this species was found in ward 17, followed closely by ward 25 where it was 12.1.

TABLE VIII

General and specific flea indices for *Rattus norvegicus* in different wards of Calcutta surveyed during the period 1936-1938

Ward number	Rats examined	Fleas found	General flea index	SPECIFIC FLEA INDICES	
				<i>Astia</i> index	<i>Cheopsis</i> index
5	16	103	6.4	2.8	3.6
7	51	356	7.0	2.6	4.4
8	53	412	7.7	3.4	4.3
6	18	23	5.3	4.0	1.3
1	28	318	11.3	7.1	4.2
11	16	151	9.4	6.0	3.4
25	40	485	12.1	10.1	2.0
16	12	61	5.1	4.8	0.3
17	38	482	12.7	10.9	1.8
18	2	8	4.0	0.5	3.5
TOTAL	274	471	9.0	5.8	3.2

With regard to the seasonal variation in the flea indices for this species of rats, there was a steady lowering of the general flea index from the beginning to the close of the year and so also the *astia* index to a certain extent. The *cheopsis*

index, however, was found to be more irregular in its variations from one period to another (see Graph)

TABLE IX

Seasonal variations in flea indices for Rattus norvegicus in Calcutta.

Period of examination	Number of <i>R. norvegicus</i> examined	<i>Astia</i> index	<i>Cheopsis</i> index
January-February 1936	44	14 0	2 1
March-April 1936	70	5 9	4 2
May-June 1936	78	5 0	4 2
July-September 1936	61	2 1	2 2
October-December 1938	21	2 8	0 9
TOTAL	274	5 8	3 2

FLEA INDICES FOR *Gunomys varius*.

In this species of rodents, the general flea index, taking all the wards together, was almost the same as that for *Rattus rattus*. But with regard to the specific flea indices, the results were more like that of *Rattus norvegicus*, the *astia* index being higher than the *cheopsis* index. This was so in all the wards surveyed without any exception (see Table X)

TABLE X

Specific flea indices for Gunomys varius in different wards of Calcutta surveyed during the period 1936-1938

Ward number	Rats examined	Fleas found	General flea index	SPECIFIC FLEA INDICES	
				<i>Astia</i> index	<i>Cheopsis</i> index
5	8	34	4 2	2 6	1 6
7	45	290	6 4	4 4	2 0
8	30	223	7 4	5 1	2 3
6	53	240	4 5	3 5	1 0
1	7	20	2 9	2 3	0 6
11	17	48	2 8	1 9	0 9
25	50	142	2 8	2 2	0 6
16					
17	3	9	2 7	2 7	0 0
18	20	140	7 0	6 6	0 4
TOTAL	233	1,145	4 9	3 7	1 2

With regard to the seasonal variation in the general and specific flea indices for this species of rats (Table XI), the indices appeared to be high during the months of January and February and then they become gradually less and less till they touched the lowest point in October at the end of the rainy season (see Graph)

TABLE XI

*Seasonal variation in the specific flea indices for Gunomys
varius in Calcutta*

Period of examination	Number of <i>G. varius</i> examined	<i>Astia</i> index	<i>Cheopsis</i> index
January-February 1936	18	5 2	2 4
March-April 1936	30	4 0	1 1
May-June 1936	77	3 7	1 3
July-September 1936	66	3 2	1 3
October-December 1938	42	3 5	0 6
TOTAL	233	3 3	2 2

FLEA INDICES FOR *Mus musculus* (HOUSE MICE)

In spite of a large number of these small rodents being trapped in different wards during the present investigations, only a limited number of them could be examined for fleas as it was only on a few occasions that these were caught singly in traps. Altogether 89 mice were examined from different wards and practically the same number of fleas were obtained on them (Table XII). *X. astia* and *X. cheopsis* were found in equal numbers. Mice for the examination of fleas were not available from wards 6 and 7.

Rat-Fleas of Calcutta.

TABLE XII

General and specific flea indices for *Mus musculus* in different wards of Calcutta surveyed during the period 1936-1938

Ward number	Number of <i>Mus musculus</i> examined	Fleas found	General flea index	SPECIFIC FLEA INDICES	
				<i>Astia</i> index	<i>Cheopsis</i> index
5	13	12	0.9	0.1	0.8
7					
8	3	7	2.3	1.3	1.0
6					
1	13	12	0.9	0.3	0.8
11	11	15	1.3	0.7	0.6
25	11	13	1.1	0.5	0.6
16	14	9	0.6	0.5	0.1
17	6	10	1.7	1.7	0.0
18	18	15	0.8	0.4	0.4
TOTAL	89	93	1.0	0.5	0.5

FLEA INDICES FOR *Bandicoota indica*

Only 7 bandicoots were examined for fleas. Most of these were from ward 25. Out of 28 fleas caught on these bandicoots 23 were *astia* and the rest *cheopsis*. Details are given in Table XIII —

TABLE XIII.

General and specific flea indices for *Bandicoota indica* in different wards of Calcutta surveyed during the period 1936-1938

Ward number	Number of <i>B. indica</i> examined	Fleas found	General flea index	SPECIFIC FLEA INDICES	
				<i>Astia</i> index	<i>Cheopsis</i> index
5	.				
7					
8	1	3	3.0	2.0	1.0
6	1	3	3.0	1.0	2.0
1					
11	1	6	6.0	5.0	1.0
25	4	16	4.0	3.8	0.2
16					
17					
18					
TOTAL	7	28	4.0	3.3	0.7

FLEA INDICES FOR *Crocidura coerulea* (MUSK RAT)

These are not rats but shrews. They do not even belong to the order of rodents. They are, however, carnivorous in habit and prey upon small mice and other kinds of rats. *X astia* and *X cheopis* were found in equal numbers on them, taking the results of all the wards surveyed together (Table XIV). In wards 16 and 17 where *astia* predominated on the other species of rodents this shrew also showed a higher *astia* index. Otherwise *cheopis* index was as high or even higher than the *astia* index in all the other wards.

TABLE XIV

General and specific flea indices for Crocidura coerulea in all the wards of Calcutta surveyed during the period 1936-1938

Ward number	Number of <i>C. coerulea</i> examined	Fleas found	General flea index.	SPECIFIC FLEA INDICES	
				<i>Astia</i> index	<i>Cheopis</i> index
5	6	16	2.7	0.7	2.0
7					
8	1	8	8.0	4.0	4.0
6	3	8	2.7	1.0	1.7
1	2	7	3.5	1.5	2.0
11	7	26	3.6	1.8	1.8
25	4	17	4.2	1.5	2.7
16	6	10	1.7	1.2	0.5
17	12	52	4.3	3.9	0.4
18	21	64	3.0	0.9	2.1
TOTAL	62	208	3.4	1.7	1.7

In a place like Calcutta, where rats are abundant, the rat-fleas, their number and species and their seasonal variations are of direct importance from an epidemiological point of view regarding plague. The interpretation of the general and the specific flea indices is, however, a complicated problem because of the variations to which these indices are subject in relation to the species of rats, the localities and the season. What we ultimately are interested in is the total number of fleas classified according to the species which are available for attacking man under suitable circumstances. Since, however, fleas cannot be independently caught, we can only obtain the index of flea catches in relation to the rodents caught. The best index for our purpose would therefore be the number of different species of fleas obtained from rodents caught in 100 traps at any given place and time. Although it was possible to obtain such an index from the work carried out, it would not have been of great use for the purposes of comparison, as similar data is not available for other plague-infected and plague-free cities or for Calcutta in the past.

When compared to other cities for which data are available, Calcutta according to the present investigations shows higher maximum and minimum flea indices.

TABLE XVII

Maximum and minimum flea indices for rodents in some of the plague-infected and plague-free cities in India

City	Province or State	Average maximum fleas per rat	Month of maximum prevalence	Average minimum fleas per rat	Month of minimum prevalence
Lucknow	United Provinces	13.0	January	1.9	August
Bombay	Bombay	5.2	March	2.5	November
Madras	Madras	5.4	January	2.2	June
Bengal	Dacca	8.2	April	2.0	October
	Calcutta*	12.0	February	3.2	August
Hyderabad	Hyderabad*	6.3	September	0.6	June

* Observations recorded during the present investigation. Flea indices for other towns mentioned in the table have been taken from the records of the British Plague Research Commission.

There was also a belief during previous years that the comparative freedom from plague enjoyed by Calcutta at present might be due to the preponderance of the fleas of the species *X. astia*, which has been shown by Hirst (*loc cit*) and others to be a less efficient vector. It is thus observed that the paucity of fleas as reported by Strickland and Roy (*loc cit*) is not borne out by the more recent survey and that the scarcity of fleas does not appear to be the explanation of the relative absence of plague in Calcutta. Neither is the flea-species factor an acceptable explanation as according to the present survey *X. cheopis* forms a fairly good proportion of rat-flea population of Calcutta and that in some wards of this city it even out-numbers *X. astia*.

SUMMARY AND CONCLUSIONS

A rat-flea survey of 10 selected wards of Calcutta was made covering a period of 12 months (January to September 1936 and October to December 1938). From the 885 rodents examined 4,888 fleas were caught, giving a flea index of 5.5. The highest flea index (9.0) was found for *Rattus norvegicus* and *Gunomys varius* came next in order with flea index of 4.9 followed closely by *Rattus rattus* with an index of 4.3. *Mus musculus*, *Bandicoota indica* and *Crocidura coerulea* showed flea indices of 1.0, 4.0 and 3.4, respectively.

Of the 4,888 fleas collected, 2,963 or 60.6 per cent belonged to the species *Xenopsylla astia* and 1,923 or 39.4 per cent to the species *Xenopsylla cheopis*. Two of the fleas belonged to species *Ctenocephalus felis*. The proportion of *astia* and *cheopis* varied not only from ward to ward but also from one species of rodent to another. *Rattus rattus* carried more *cheopis* than *astia*, while the reverse was the case with *Rattus norvegicus* and *Gunomys varius*.

It is suggested that the distinctly higher general and the specific flea indices found during the present observations as compared with previous findings have been in all probability due to better technique and control over the collection of rats and fleas than to any actual differences in the prevalence of fleas in the city at different periods.

Compared with other Indian cities, Calcutta possesses high general and specific flea indices and this flea factor is quite favourable for the spread of plague in an epidemic form, provided the other factors concerned in the epidemiology of plague are also favourable.

ACKNOWLEDGMENT

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FURTHER STUDIES ON THE INFLUENCE OF PYROPHOSPHATE ON THE OXIDATION OF VITAMIN C

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THE protective action of pyrophosphate against the oxidation of vitamin C was first demonstrated by Giri (1937), who found that pyrophosphate exerts a stabilizing action on the vitamin both in the presence and absence of Cu and in buffered solutions of the vitamin. In a subsequent communication Giri and Doctor (1938) indicated the use of pyrophosphate for preventing the oxidation of the vitamin during extraction from plant and animal tissues, and the reagent has since been used in the estimation of the vitamin C content of animal tissues by Giri (1939), and in fruits and vegetables by Mitra, Mitra and Roy (1940). The use of pyrophosphate in the preparation of stable solutions of vitamin C was also indicated. It was also found by Giri (1937) that pyrophosphate exerts a stabilizing action on the vitamin contained in lemon juice. Klodt and Stieb (1938) confirmed the observation that pyrophosphate exerts a stabilizing action on the vitamin contained in lemon juice. They found that, while the diluted lemon juice loses completely its vitamin C potency after four days, the addition of pyrophosphate to lemon juice in such low concentration as 0.05 per cent preserved the potency of the vitamin for a much longer period, and even after 30 days, 16 per cent of the original vitamin was present in the juice. The authors suggested that the stability of the vitamin

present in living tissues can be increased by the addition of small quantities of pyrophosphate. In the present investigation the protective action of pyrophosphate on the oxidation of vitamin C by catalysts like Cu, Fe, Norite and the enzyme ascorbic acid oxidase has been studied.

EXPERIMENTAL

The rate of oxidation of vitamin C was measured by titration with the indophenol dye. In some cases the results obtained by the titrimetric method were repeated by measuring the rate of oxidation of the vitamin manometrically in Warburg's respirometers.

The manometric method consisted in measuring the O_2 uptake from a solution of ascorbic acid, shaken in air in Warburg manometers with 0.2 c.c. of 20 per cent KOH and filter-paper in the central chamber. The buffer and the catalyst—Cu or the enzyme, ascorbic acid oxidase—together with pyrophosphate were placed in the main chamber of the vessels, ascorbic acid solution being kept in the side arm and dropped into the main vessel when temperature equilibrium was reached, and readings were taken at definite intervals of time.

The water used for the preparation of buffers and other solutions was twice distilled in a Pyrex distillation apparatus.

Pyrophosphate solution was prepared by dissolving the salt in the minimum amount of water, and neutralizing with acetic acid to the desired pH. The pH of the solution was always tested before addition to the reaction mixture.

(2) *Influence of different samples of pyrophosphate on the oxidation of vitamin C*—In previous investigations the pyrophosphate was prepared from Sørensen's secondary sodium phosphate, by heating the salt in platinum crucibles to red heat. In view of the fact that different samples of secondary sodium phosphates may possess varying degrees of purity, the protective properties of pyrophosphate prepared from different samples of sodium phosphate were studied both in buffered solutions of the vitamin and in the presence of trichloroacetic acid, which is widely used for the extraction of the vitamin from plant and animal tissues.

Three samples of pyrophosphate were tested —

Pyrophosphate A—prepared from Sørensen's secondary sodium phosphate

Pyrophosphate B—prepared from Merck secondary sodium phosphate (cryst.)

Pyrophosphate C—crystalline salt $Na_4P_2O_7 \cdot 10H_2O$

The reaction mixtures contained 20 c.c. of 5 per cent trichloroacetic acid and 5 c.c. of vitamin C solution, containing 5 mg. of the vitamin. The concentration of pyrophosphate in the reaction mixture amounted to 0.8 per cent anhydrous $Na_4P_2O_7$.

The results are presented in Table I —

TABLE I

Protective action of the pyrophosphates against the oxidation of vitamin C in trichloroacetic acid

	OXIDATION OF VITAMIN C AT 37°C				
	Mg of vitamin C in the total volume of the reaction mixture Time in hours				
	0	1	4	24	48
1 Vitamin C + trichloroacetic acid	5.0	4.5	2.1	0.10	0.0
2 Vitamin C + trichloroacetic acid + pyrophosphate A	5.0	5.0	4.8	4.0	2.2
3 Vitamin C + trichloroacetic acid + pyrophosphate B	5.0	5.0	4.8	3.0	1.6
4 Vitamin C + trichloroacetic acid + pyrophosphate C	5.0	5.0	4.8	2.4	1.0

The protective action of these pyrophosphates was also tested in phosphate buffered solutions. The reaction mixtures contained 20 cc of M/15 phosphate buffer (pH 7.1) and 5 mg vitamin C. The concentration of pyrophosphate was adjusted to 0.66 per cent of the anhydrous salt. The total volume of the reaction mixture was 30 cc.

TABLE II

Protective action of the pyrophosphates against the oxidation of vitamin C in buffer solutions

	OXIDATION OF VITAMIN C AT 37°C			
	Mg of vitamin C in the total volume of the reaction mixture Time in minutes			
	0	30	60	120
1 Vitamin C	5.0	3.8	3.0	1.6
2 Vitamin C + pyrophosphate A	5.0	4.8	4.7	4.5
3 Vitamin C + pyrophosphate B	5.0	4.8	4.7	4.5
4 Vitamin C + pyrophosphate C	5.0	4.7	4.7	4.3

74 *Influence of Pyrophosphate on the Oxidation of Vitamin C*

The degree of protection exerted by the pyrophosphates against the oxidation of the vitamin in the presence of trichloroacetic acid varied slightly with the type of pyrophosphate used. This difference was, however, not so pronounced in phosphate buffer solutions. Among the pyrophosphates studied, that prepared from Sørensen's secondary sodium phosphate exerted the maximum protective action.

The pyrophosphate A was, therefore, used in all subsequent investigations.

(iv) *Influence of pyrophosphate on the oxidation of added vitamin C in urine* — Vitamin C in urine is easily oxidized, and in determining vitamin C excretion it is necessary to prevent the destruction of the vitamin before analysis. It was, therefore, of interest to know whether pyrophosphate can be used for the stabilization of the vitamin in urine.

To 50 c.c. of urine were added 10 c.c. of water and 5 c.c. of vitamin C solution containing 5 mg. of the vitamin. To one of the samples pyrophosphate was added in such amounts as to bring the total concentration of the salt to 0.64 per cent. The rate of oxidation of the added vitamin in urine was determined at room temperature. The results are presented in Table III —

TABLE III

<i>Influence of pyrophosphate on the oxidation of added vitamin in urine</i>				
	OXIDATION OF VITAMIN C AT 37°C			
	Mg. of vitamin C in the total volume of the urine Time in hours			
	0	1	18	24
1 Urine + vitamin C	10	9.5	3.8	1.9
2 Urine + vitamin C + pyrophosphate 0.64 per cent	10	9.5	6.3	5.0

The results show that pyrophosphate protects the added vitamin C against oxidation.

The effect of addition of pyrophosphate to urine in presence of trichloroacetic acid, was also tried, and it was found that pyrophosphate exerted similar protection against the oxidation of the vitamin in urine.

(v) *Influence of pyrophosphate on the oxidation of vitamin C by ferrous iron* — Pyrophosphate is an inhibitor of many iron catalysed reactions. It has been shown

to form stable complexes with ferric ions (Warburg, 1927) The influence of pyrophosphate on the catalytic oxidation of the vitamin by ferrous ions was, therefore, tested

The reaction mixture consisted of 10 c c of acetate buffer (pH 5.6) and 5 mg of the vitamin and 0.7 mg of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ in the total volume of the reaction mixture. The total concentration of pyrophosphate was adjusted to 1 per cent. The results are presented in Table IV —

TABLE IV

Influence of pyrophosphate on the oxidation of vitamin C by ferrous iron

		OXIDATION OF VITAMIN C AT 57°C			
		M ₂ of vitamin C in the total volume of the reaction mixture Time in minutes			
		0	10	20	60
1. Vitamin C	..	50	47	25	20
2. Vitamin C — Fe ⁺⁺		50	25	20	21
3. Vitamin C — Fe ⁺⁺ — pyrophosphate		50	50	48	45

The table shows that pyrophosphate exerts a protective action against the oxidation of the vitamin by ferrous iron.

(iv) *Influence of pyrophosphate on the oxidation of vitamin C by Norite.*—Fox and Levy (1933) have shown that Norite charcoal rapidly oxidizes ascorbic acid into dehydroascorbic acid. As it has been shown that the catalytic activity of charcoal is related to its iron content, it was of interest to know whether pyrophosphate exerts a protective action against the oxidation of the vitamin by charcoal.

The reaction mixture contained acetate buffer (pH 5.6) and 5 mg. of vitamin C, the total volume amounting to 10 c.c. of 0.5 g. Norite was used for the oxidation of the vitamin. The concentration of pyrophosphate in

the reaction mixture was adjusted to 1 per cent The results are presented in Table V —

TABLE V

Action of pyrophosphate on the oxidation of vitamin C by Norite

	OXIDATION OF VITAMIN C AT 37°C				
	Mg of vitamin C in the total volume of the reaction mixture				
	Time in minutes				
	0	10	20	30	60
1 Vitamin C	5.0	4.3	4.0	3.5	2.9
2 Vitamin C + Norite	5.0	3.0	2.2	2.0	1.4
3 Vitamin C + Norite + pyrophosphate	5.0	3.5	3.1	3.0	2.6

The results show that pyrophosphate protects the vitamin from Norite oxidation

(v) *Influence of pyrophosphate on the oxidation of vitamin C by ascorbic acid oxidase* —Stotz, Harrer and King (1937a, b), on the basis of their observations on the action of a number of copper inhibitors on the activity of the enzyme ascorbic acid oxidase, conclude that the activity of the enzyme, previously ascribed to a specific oxidase, is due to the copper present in combination with protein material. They found that diethyldi-thiocarbamate, 8-hydroxy-quinoline, pyridine, potassium thiocyanate, sodium cyanide, potassium ethyl xanthate, potassium ferrocyanide and sodium sulphide produced nearly complete inhibition of the activity of the enzyme, as well as copper and copper-protein mixtures. It was, therefore, of interest to know whether pyrophosphate which inhibits the Cu oxidation of the vitamin, exerts the same degree of inhibition on the enzymic oxidation of the vitamin.

Preparation of ascorbic acid oxidase —The enzyme was prepared from the juice of the pericarp of *Cucumis sativus*, by precipitation with acetone. The juice was obtained by pressing the pulp with cloth and the enzyme was precipitated from the

freshly expressed juice by adding an equal volume of acetone, and centrifuging to remove the precipitate, which was re-dispersed in the same volume of water as the original juice

The activity of the enzyme was studied at pH 5.6 in the absence and in the presence of sodium pyrophosphate both by measuring the oxygen consumption in Warburg manometers and titrimetrically by the dye method. For the titrimetric method the reaction mixtures in each case consisted of 20 c.c. of M/5 acetate buffer (pH 5.6), 5 c.c. of vitamin C solution containing 5 mg. of the vitamin. The concentration of pyrophosphate was adjusted to 1 per cent, the total volume of the solution being adjusted to 30 c.c. The vitamin was estimated at stated intervals by titration with the indophenol dye. The results are presented in Table VI —

TABLE VI

Influence of pyrophosphate on the enzymic oxidation of vitamin C

	OXIDATION OF VITAMIN C AT 37°C				
	Mg. of vitamin C in the total volume of the reaction mixture				
	Time in minutes				
	0	10	20	30	60
1 Vitamin C + enzyme	5.0	2.9	2.3	2.0	1.4
2 Vitamin C + enzyme + 1 per cent pyrophosphate	5.0	2.9	2.5	2.2	1.4

The experiment was repeated with the Warburg apparatus, and the rate of oxidation was followed by measuring the oxygen uptake. The vessels were air filled and contained 2 mg. of vitamin C and 1 c.c. of M/5 acetate buffer (pH 5.6), 0.5 c.c. of enzyme or copper sulphate solution containing 0.07 mg. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, pyrophosphate concentration was varied. The total volume of the mixture in the main vessel was 3 c.c. The oxygen uptake was measured after the temperature equilibrium was reached. The results are presented in Graphs 1 and 2.

The results of a typical experiment presented in Table V show that pyrophosphate has no protective action against the enzymic oxidation of vitamin C. A repetition of the experiment carried out manometrically has shown that pyrophosphate exerts very slight protection at 0.83 per cent concentration against the enzymic oxidation of the vitamin. But the degree of protection exerted is very little compared to that of the protection exerted against Cu oxidation of the

(iv) *Effect of pyrophosphate on the oxidation of vitamin C by albumin copper complex*—Stotz *et al* (1937a, b) have shown that a mixture of copper and albumin exhibits the characteristic properties of the enzyme ascorbic acid oxidase. It was, therefore, of interest to investigate the protective action of pyrophosphate against the oxidation of the vitamin by Cu -albumin complex.

The Cu -albumin complex was prepared by dissolving 50 mg of egg albumin in 10 c c of water, and to this was added 10 c c of a solution containing 0.345 mg of CuSO_4 per c c. The mixture was well shaken before use. The reaction mixtures consisted of 20 c c of acetate buffer, 5 mg vitamin C, the total volume being adjusted to 31 c c. Two c c of the Cu -albumin mixture was used for oxidation of the vitamin. The concentration of pyrophosphate was adjusted to 1.0 per cent. The results are presented in Table VII —

TABLE VII

Effect of pyrophosphate on the oxidation of vitamin C by Cu -albumin complex

	OXIDATION OF VITAMIN C AT 37°C				
	Mg of vitamin C in the total volume of the reaction mixture				
	Time in minutes				
	0	10	20	30	60
1 Vitamin C	5.0	4.1	3.6	3.3	2.6
2 Vitamin C + Cu albumin	5.0	2.7	2.3	1.9	0.42
3 Vitamin C + Cu albumin + pyrophosphate	5.0	3.6	3.2	2.9	2.2

The results show that pyrophosphate exerts a protective action against the oxidation of the vitamin by the Cu -albumin complex.

DISCUSSION

The rôle of pyrophosphate in relation to respiration mechanisms, and other oxidation-reduction systems in living tissues, has been the subject of intense study. Dixon and Elliott (1929) have shown that pyrophosphate acts as a respiratory inhibitor. Unlike cyanide inhibition, which is mainly due to its action on the cytochrome oxidase, pyrophosphate inhibition of respiration is not due to its effect on cytochrome oxidase, as it has no action on this enzyme. Leloir and Dixon (1937) investigated the effect of pyrophosphate on the dehydrogenase and found that

pyrophosphate strongly inhibits the succinic dehydrogenases, without any effect on the other dehydrogenases investigated. Thus pyrophosphate is shown to act as a respiratory inhibitor by inhibiting the activity of succinic dehydrogenase which according to Szent-György (1935) plays an important rôle in the respiration of animal tissues.

It is reported by Green (1936) that pyrophosphate causes 100 per cent acceleration of malic dehydrogenase, while the lactic dehydrogenase is not affected. Peters and Sinclair (1933) have shown that sodium pyrophosphate added with lactate increased the respiration rate of nunced pigeon brain in phosphate buffer to a considerable extent. Peters *et al* (1933, 1935) found that pyrophosphate (M/100) increased the catatorulin effect of vitamin B₁ in the metabolism of lactate and pyruvate. Recently, Ochoa (1939) has shown that pyrophosphate inhibits the cocarboxylase synthesis. Greig and Munroe (1939) investigated the effects of pyrophosphate on respiring slices of different tissues, and found that it produced various effects on the metabolism of tissue slices depending both on the tissue and the substrate employed. Pyrophosphate has thus varied effects on respiration and other oxidation-reduction systems in living tissues.

The present investigation on the action of pyrophosphate on the oxidation of vitamin C has shown that it protects not only the Cu oxidation of the vitamin, but also its oxidation by iron, Norite, and Cu-albumin complex.

It would perhaps be appropriate here to explain certain discrepancies observed by Klodt and Stieb (*loc cit*) in their studies on the stabilization of vitamin C by pyrophosphate. The authors found that the vitamin present in lemon juice is protected from oxidation in presence of small concentrations of pyrophosphate (0.05 per cent), while pure synthetic ascorbic acid solutions are not stabilized by the reagent. This may be explained as being due to the lack of control in pH of the solutions. It is so well known that pyrophosphate solutions are intensely alkaline in reaction and the solution should be neutralized to the desired pH before it is added to the reaction mixture. It is clear from their experiments that the pyrophosphate was added to ascorbic acid without neutralizing the solution. In the absence of buffer, the pH would be shifted from acid to highly alkaline range when pyrophosphate is added to ascorbic acid solutions. Their interpretation of the data relating to pure synthetic ascorbic acid solution is, therefore, at fault because changes in reaction caused by the addition of pyrophosphate were not given consideration. In other words, there was no pH control. In the case of lime juice, however, the addition of pyrophosphate might not have altered the pH, as a result of the high acidity and buffering capacity of the juice, and hence the protection is observed, because changes in reaction caused by the addition of pyrophosphate is negligible. It would, therefore, be entirely erroneous to conclude from observations made without any proper control of pH, that pyrophosphate does not protect the vitamin in pure solutions.

Much controversy has raged round the question of the chemical nature of ascorbic acid oxidase. Stotz *et al* (*loc cit*) have put forward the view that the enzyme is a copper protein compound and that the activity of ascorbic acid oxidase is related to the presence of copper in combination with proteins. Spruyt and

Vogelsang (1938), on the other hand, have shown that no parallelism exists between the oxidase action and copper content, and that the enzymic oxidation cannot be ascribed to the presence of copper alone. The results of the present investigation indicate that the copper oxidation of the vitamin is greatly inhibited by pyrophosphate, while the enzymic oxidation is not much affected by pyrophosphate of the same concentration (Graphs 1 and 2). It would appear, therefore, that either Cu does not constitute the active component of the enzyme, or the Cu is present in a complex combination with a protein or any similar carrier, which may not be similarly influenced by pyrophosphate in the same way as the Cu ion. Experiments on the behaviour of a Cu-albumin complex towards vitamin C have shown that pyrophosphate protects oxidation by the Cu-protein complex. Thus, the behaviour of pyrophosphate towards Cu, Cu-albumin complex on the one hand and the enzyme on the other, differs from that of other inhibitors as reported by Stotz *et al* (*loc cit*) in that the latter inhibit the enzymic oxidation, while pyrophosphate does not. It is impossible at present fully to explain the experiments and until more is known concerning the nature of the Cu-combination, theories about the metal protein combination of the enzyme ascorbic acid oxidase must be accepted with caution.

SUMMARY

The effect of pyrophosphate on the oxidation of vitamin C by various catalysts is described. It was found that pyrophosphate exerts protective action on the oxidation of the vitamin by Cu, Fe, Norite and copper-albumin complex. The enzymic oxidation was, however, very little affected by pyrophosphate. It also exerted protective action against the oxidation of added vitamin C in urine. The bearing of these results on the nature of ascorbic acid oxidase is discussed.

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THE EFFECT OF WASHING AND COOKING ON THE NICOTINIC ACID CONTENT OF RAW AND PARBOILED RICE

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PREVIOUS investigations in this laboratory (Aykroyd, Krishnan, Passmore and Sundararajan, 1940, Aykroyd and Swaminathan, 1940) have shown that the par-boiling of rice (i.e. steaming in the husk) greatly reduces losses of vitamin B₁ and nicotinic acid from the grain on milling. Parboiled milled rice contains 3 to 4 times as much of these factors as raw rice of the same botanical variety milled to the same degree. The effect of the washing and cooking of rice on vitamin B₁ and nicotinic acid was also studied, and it was found that on the average these processes cause a loss of 40 to 50 per cent. There was some indication that vitamin B₁ and nicotinic acid are more easily removed by washing and cooking from raw than from par-boiled rice, but sufficient data were not obtained to establish this point. In the present investigation a more detailed study of losses of nicotinic acid under these conditions was carried out. The distribution of nicotinic acid in the rice grain and its behaviour during the process of parboiling are similar to those of vitamin B₁, i.e. nicotinic acid is most abundantly present in the germ and pericarp, while the starchy endosperm contains it in much smaller quantities, during parboiling it diffuses through the endosperm and cannot be removed on subsequent milling (Aykroyd and Swaminathan, *loc cit*). It is easier to assay nicotinic acid chemically than vitamin B₁. Hence it was felt that a study of the effect of washing and cooking on the nicotinic acid content of rice would provide a clue to the effect of these processes on vitamin B₁ content.

EXPERIMENTAL

Ordinary commercial samples of raw milled and parboiled milled rice were obtained from the local market. These included several different varieties. The

84 *Effect of Washing and Cooking on Nicotinic Acid Content of Rice*

nicotinic acid content of the samples as purchased was determined. Subsequently the rices were washed and cooked according to practices commonly followed in Indian homes. Rice is usually washed several times in preparation for consumption. The nicotinic acid determinations were carried out on the water used in washing, the cooking water and the rice after washing and cooking. The percentage loss of nicotinic acid from raw and parboiled rice so treated was compared.

Washing and cooking were carried out as follows. Two hundred grammes of rice were washed three times, usually with about its own weight of water, the rice being stirred under water for two minutes with a glass-rod for each washing, and the washings decanted off as completely as possible. The washed rice was then added to 1 litre of boiling water and cooked until fit for consumption (20 minutes in the case of raw rice and 30 minutes in the case of parboiled rice), care being taken to prevent evaporation of water as much as possible. The cooking water (conjee) was then decanted off and measured.

Estimation of nicotinic acid—The cyanogen bromide-aniline method as developed in this laboratory (Swaminathan, 1938) was used for the estimation of nicotinic acid in the various samples. In carrying out tests on the water used for washing and cooking, the following procedure was adopted —

Five ml of lead acetate (30 per cent solution) were added and the precipitate removed by centrifuging. Excess of lead was removed as sulphate. The clear solution was adjusted to pH 6 and evaporated to a small bulk (75 ml) on a water-bath or directly over a flame in big beakers. It was again adjusted to pH 1 by the addition of enough sulphuric acid and filtered or centrifuged to remove insoluble matter, consisting mostly of starch and some lead sulphate. The test was then applied to this solution.

RESULTS

The results of experiments with three samples of raw and parboiled rice respectively are shown in Table I. Data obtained in an earlier investigation are also included in this table. The same amounts of water were used in the washing and cooking of all the samples.

It will be seen that the average loss of nicotinic acid from the raw samples on washing was 50 per cent, whereas in the case of the parboiled samples it was only 10 per cent. The losses on cooking from already washed samples of raw and parboiled rice were on the average 40 and 45 per cent respectively, i.e. of the same order in each case. It is to be observed that 'conjee' from parboiled rice contains 2 to 3 times as much nicotinic acid as the 'conjee' from raw rice. The percentage losses from the raw and parboiled samples, caused by both washing and cooking, were about 70 and 50 per cent respectively.

The figures given in Table I indicate that the losses on washing are quantitatively greater than those occurring on cooking. The effect of washing in five samples

TABLE I.

The effect of washing and cooking on the nicotinic acid content of raw and parboiled rice

Rice sample	Unwashed rice mg/100 g	Wash water removed from 100 g of rice (mg)	Percentage lost in wash water	Washed rice (by difference)	Cooked rice mg/100 g of original rice	Conjee (water used for cooking)	Percentage of nicotinic acid present in washed rice removed on cooking
1 Raw hand pounded	25	11	44	14	07	06	45
2 „ milled	17	07	41	10	06	04	40
3 „ „	16	10	63	06	04	02	33
	Average		50				40
4 Parboiled hand pounded	33	03	10	30	14	15	50
5 „ milled	30	03	10	27	13	15	55
6 „ „	30	03	10	27	19	09	30
	Average		10				45

86 *Effect of Washing and Cooking on Nicotinic Acid Content of Rice*

of raw and five samples of parboiled rice was then investigated, the results being given in Table II —

TABLE II

The effect of washing on the nicotinic acid content of raw milled and parboiled milled rice

Rice sample number	NICOTINIC ACID LOST IN WASH WATER					Percentage lost in wash water	Washed rice mg /100 g of original rice
	Unwashed rice mg /100 g	1st washing	2nd washing	3rd washing	Total		
Raw milled { 1 2 3 4 5	1.6	0.93	0.10	0.02	1.05	66	0.55
	2.0	1.00	0.18	0.04	1.31	66	0.69
	1.5	0.64	0.15	0.12	0.91	60	0.50
	1.3	0.60	0.06	0.03	0.69	53	0.61
	1.4	0.50	0.12	0.06	0.77	55	0.63
Average	1.6				0.95	60	0.65
Parboiled milled { 1 2 3 4 5	3.0	0.15	0.11	0.05	0.31	10	2.69
	3.1	0.36	0.15	0.10	0.61	20	2.40
	3.9	0.20	0.05	0.04	0.29	7	3.61
	3.0	0.18	0.05	0.03	0.26	9	2.74
	3.1	0.22	0.08	0.08	0.38	12	2.72
Average	3.2				0.37	12	2.83

Table II shows that on the average about 60 per cent of the nicotinic acid present in the raw rice samples was washed out. The first washing removed most of the nicotinic acid, the second and third washings containing but little. On the other hand, the parboiled milled rice samples, which originally contained twice as much nicotinic acid as the raw milled samples, lost on the average only 12 per cent of their nicotinic acid. Even washing thrice with four times its weight of water failed to remove more than 16 per cent of the nicotinic acid contained in parboiled rice, as will be seen from Table III —

TABLE III

The effect of washing with varying amounts of water on the nicotinic acid content of raw milled and parboiled rice

Rice sample number	Unwashed rice mg /100 g	Quantity of water used for washing	NICOTINIC ACID LOST IN WASHINGS				Percentage lost in washings	Washed rice mg /100 g of original rice
			1	2	3	Total		
Raw milled I	1.6	Equal	0.93	0.10	0.02	1.05	66	0.55
" I	1.6	4 times	1.03	0.04	0.01	1.08	68	0.53
Parboiled milled I	3.0	Equal	0.15	0.11	0.05	0.31	10	2.69
" I	3.0	4 times	0.23	0.14	0.09	0.46	15	2.54

DISCUSSION

The losses of water-soluble vitamins to the consumer which are caused by cooking *per se* largely depend on the amount of cooking water used and whether it is discarded or not. If only just enough water to cover the rice is used in cooking, losses will be small since most of the water is consumed. Methods of cooking rice in India vary in different communities and districts, it is often the practice to use as little water as possible and the 'conjee' is often taken. On the other hand, rice is usually washed before cooking, and washing is regarded as essential by the rice-eating population. The losses from washing are thus more important and constant than the losses from cooking.

The parboiling of rice, in addition to reducing losses of nicotinic acid on milling, has also the effect of reducing losses occurring when rice is washed according to ordinary domestic practice. It may be assumed that the vitamin B₁ content of raw and parboiled rice will be affected in the same way by washing. It has been shown (Aykroyd *et al*, *loc cit*) that in India beriberi is common in districts in which

the poorer classes consume a diet largely composed of raw milled rice, whereas the consumers of milled parboiled rice rarely contract the disease. In connection with the beriberi problem, parboiled milled rice has two advantages over raw milled rice (a) it has a higher initial vitamin B₁ content, and (b) the vitamin B₁ present in the former is more difficult to wash out. Both are of importance in producing the almost complete immunity of the parboiled rice eater to beriberi. Platt (1939) has shown that the washing of raw rice is a significant factor in the causation of beriberi in China.

The fact that parboiled rice loses a smaller proportion of its nicotinic acid on washing than raw rice is presumably due to the diffusion of nicotinic acid through the endosperm on parboiling. In raw rice most of the nicotinic acid (and vitamin B₁) is present in the outer layers. When rice is washed in the cold the water does not pass right through the grain. On the other hand, boiling water used for cooking, which penetrates the grain completely, removed about the same proportion of nicotinic acid from already washed raw rice and parboiled rice.

SUMMARY

1 The effect of washing and cooking on the nicotinic acid content of some commercial samples of rice has been studied.

2 Raw milled rice loses the greater part of its nicotinic acid (60 per cent) during the process of washing, while parboiled milled rice, in spite of its higher nicotinic acid content, loses much less (12 per cent). Washed parboiled milled rice contains on the average 4 times as much nicotinic acid as washed raw milled rice. These figures and percentages are probably applicable in the case of vitamin B₁.

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THE VITAMIN B₁ CONTENT OF THE MILLETS *ELEUSINE*
CORACANA AND *SORGHUM VULGARE*, WHOLE
WHEAT GROWN UNDER DIFFERENT
MANURIAL CONDITIONS, AND RICE
STORED UNDERGROUND

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DETAILED investigations on the vitamin B₁ content of rice have recently been reported from these laboratories (Aykroyd, Krishnan, Passmore and Sundararajan, 1940). While the quantity of rice consumed in India exceeds that of all other cereals put together, wheat and millet are also extensively cultivated. There are some 20 million acres under *Sorghum vulgare*, called jowar in Northern India and cholam in the South. *Eleusine coracana* (ragi) is also an important crop, being the principal staple of the Mysore tableland and widely grown elsewhere in South India. In 1938 there were some 3.8 million acres in British India under this grain.

The application of the thiochrome method of assaying vitamin B₁ to cereals has already been described (Aykroyd *et al*, *loc cit*). In the present investigation the vitamin B₁ content of 10 strains of cholam and 6 strains of ragi respectively was determined by this method. Vitamin B₁ assays of wheat subjected to various kinds of manurial treatment, and of rice stored underground for 2 months, are also reported.

RESULTS

The results of the tests on cholam and ragi are given in Tables I and II respectively. The samples were obtained from the Millets Specialist, Government

Agricultural College, Coimbatore The average values obtained for cholam and ragi were 3.5 µg/g and 4.2 µg/g respectively. There was some variation from sample to sample, and among the samples of ragi one cross (EC 1540) stood out as being particularly rich in vitamin B₁. The essential point, however, is that all the strains contained more than enough vitamin to prevent beriberi if consumed as the principal ingredient in the diet. The average vitamin B₁/calorie ratios were 1.2 in the case of ragi and 1.0 in the case of cholam. The work of Williams and Spies (1938) suggests that it is only when the vitamin B₁/calorie ratio of diets falls below 0.250 that there is danger of the occurrence of beriberi.

The vitamin B₁ content of these millets is of the same order as that of shelled rice, i.e. rice with germ and pericarp intact. But rice before consumption is subjected to various processes—pounding, milling and washing—which reduce vitamin B₁ content (Aykroyd *et al*, *loc cit*). Ragi and cholam, on the other hand, are usually consumed 'whole', and are not washed before cooking. The vitamin B₁ content of diets based on rice can thus be considerably raised by the inclusion of some millet in place of an equivalent quantity of rice. Such an addition or supplement is particularly valuable when the diet is composed chiefly of raw milled rice, which contains much less vitamin B₁ than parboiled milled rice. To the best of the authors' knowledge, beriberi does not occur among those who consume cholam or ragi as their staple cereal.

TABLE I

Vitamin B₁ content of different strains of cholam (Sorghum vulgare)

Serial number	Number used by Agric. Dept (strain number)	Local name of variety	Where grown normally	Colour of the grain	Irrigated or rain fed	Vitamin B ₁ µg/g
1	AS 29	Periamanjai	Coimbatore	Yellow	Rain fed	3.1
2	AS 1093	Talaivirichan	"	White	"	4.9
3	T/1	Tella jonna	Bellary	"	"	3.5
4	N 29/68	Patcha jonna	Nandyal	Yellow	"	2.7
5	AS 2095	Vellai cholam	Coimbatore	White	Irrigated	3.7
6	AS 389	Sen cholam	,	Red	"	2.8
Mean						3.5

TABLE II

Vitamin B₁ content of different strains of ragi (Eleusine coracana)

Serial number	Number used by Agric Dept (strain number)	Local name of variety	Where grown normally	Colour of the grain	Time from sowing to harvest (days)	Vitamin B ₁ µg /g
1	EC 24		Coimbatore	Brown	135	3.8
2	EC 47		Bellary	„	135	3.5
3	EC 297	T/S ragi	„	„	135	4.5
4	EC 593	Gidda Aryan	Salem	„	120	3.7
5	EC 3517	Mutti ragi	Coimbatore	„	110	4.0
6	EC 1507	Poorasi keraru	South Arcot	„	105	4.0
7	EC 3735	} Derived from crosses at the Millets Breeding Station, Coimbatore }		„	110	4.7
8	EC 3030			„	110	4.2
9	EC 1540			White	130	6.5
10	EC 2928			„	130	2.7
Mean						4.2

The vitamin B₁ content of wheat—Samples of wheat grown under various conditions at the Government Agricultural College, Lyallpur, Punjab, were assayed, the results being given in Table III. Each treatment was applied to 4 plots and the samples investigated were composite samples obtained from the 4 plots. The strain of wheat was the same throughout. The values obtained for the various samples did not show important variations, suggesting that the vitamin B₁ content of wheat, and presumably of other cereals, is little influenced by soil conditions.

TABLE III

*Vitamin B₁ content of wheat grown under different manurial conditions
Each is a composite of 4 samples grown in 4 different plots*

Serial number	Manurial treatment	Vitamin B ₁ $\mu\text{g/g}$
1	No manure	3.6
2	Potassium nitrate alone @ 60 lb N per acre	4.0
3	, , + green manure @ 5 tons per acre	3.6
4	Superphosphate alone @ 60 lb P per acre	3.3
5	, + ammonium sulphate @ 60 lb N per acre	4.3

Harris (1934), investigating the vitamin B₁ content of samples obtained from the Rothamstead Experimental Station, arrived at a similar conclusion. Leong (1939) has recently made a further study of this question and has recorded the following values for the vitamin B₁ content of wheat grown at the Rothamstead Experimental Station on plots treated in different ways —

Treatment	Vitamin B ₁ content $\mu\text{g/g}$
14 tons of dung per acre	3.6
No manure	3.0
Complete mineral manure	3.9
Complete mineral manure + 412 lb of ammonium sulphate per acre	3.6
412 lb of ammonium sulphate per acre	3.6

Leong's figures, obtained by the bradycardia method of assay, are of the same order as those shown in Table III and give no indication that the vitamin B₁ content of cereals can be raised by appropriate treatment of the soil. Essentially the same result was recorded by Leong in the case of samples of barley. Scheunert and Schieblisch (1936) and Scheunert and Wagner (1937) reported that differences in soil conditions did not affect the vitamin B₁ content of wheat, rye or barley.

Whole-wheat eaters do not suffer from beriberi, and the inclusion of some whole wheat in the diet of consumers of raw milled rice would be preventive of the disease.

The vitamin B₁ content of rice stored underground—Rice from freshly harvested paddy is not usually consumed in India in the raw state, i.e. without parboiling. Unmatured raw rice cooks to a pasty consistency and is said to be difficult to digest. The parboiling of newly harvested paddy, on the other hand, makes the rice obtained from it immediately fit for consumption, and in areas in which raw rice is generally preferred it is often the custom to use parboiled rice for one or two months after the harvest, because this makes the new crop immediately available. If the grain is to be consumed without previous parboiling, the paddy is usually stored for several months, after which the pounded or milled rice obtained from it is considered fit for consumption. To quicken the process of maturing or 'curing', paddy is often stored underground in a pit for 2 months or more. There is a belief that storage under these conditions improves the nutritive value of rice and it was suggested to us that it might have the same effect as parboiling, which causes the vitamin B₁ contained in the germ and pericarp to diffuse inwards into the endosperm, so that the vitamin cannot be removed by milling.

Two varieties of paddy, CO 5 and ADT 11, grown in the Trichinopoly district, were stored underground for two months and subsequently milled in a local mill to the ordinary commercial degree. The vitamin B₁ content of the husked grain with germ and pericarp intact and the milled product obtained from these samples was determined. The results are shown in Table IV—

TABLE IV

Vitamin B₁ content of whole and milled rice from paddy stored underground for two months after harvest

Serial number	Variety of paddy	Vitamin B ₁ µg/g	Percentage of vitamin B ₁ remaining after milling
1	ADT 11 whole	2.4	33
2	ADT 11 milled	0.8	
3	CO 5 whole	3.2	38
4	CO 5 milled	1.2	

It will be seen that milling removed about two-thirds of the vitamin B₁ present in the unhusked rice. This corresponds to the proportion generally removed in the milling of raw rice, in the case of parboiled rice the loss on milling is usually about one-third (Aykroyd *et al*, *loc cit*). The values given by these samples

correspond to those given by husked and milled raw rice generally. It is therefore evident that storage underground does not produce the effect of parboiling and the milled product obtained from paddy matured in this way is no richer in vitamin B₁ than ordinary commercial raw milled rice.

SUMMARY

1 Samples of *Eleusine coracana* and *Sorghum vulgare* were found to have an average vitamin B₁ content of 4.2 and 3.5 micrograms per gramme respectively. The thiochrome method of assay was used.

2 Samples of wheat grown under different manurial conditions were approximately similar in vitamin B₁ content.

3 Rice milled from paddy stored underground did not contain more vitamin B₁ than ordinary commercial raw milled rice.

ACKNOWLEDGMENTS

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THE VITAMIN A CONTENT OF SOME INDIAN FISH-LIVER OILS

BY

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[Received for publication, July 8, 1940]

SEVERAL investigations on the vitamin A content of the liver oils of Indian marine and fresh-water fish have already been reported [Chakravorty, Mookerji and Guha, 1933, Datta and Banerjee, 1934, Ghosh and Guha, 1935, De (N K), 1935a, 1937, De (N K), Majumdar and Sundararajan, 1938, Basu and De (H N), 1938, Seshan, 1940, Basu, Sircar and Gupta, 1940]. In the present paper the results of the spectrophotometric assay of 35 samples of marine fish-liver oil are reported. The method followed was that originally described by De (N K) (1935b, 1937). Nearly all the samples were sent to the laboratories by the Director of Fisheries, Government of Madras. All were obtained on the South-West Coast. The oil was extracted by boiling the livers in an open copper vessel with a lining of tin. After extraction, the oil was washed with hot water and subsequently filtered. The results are given in the Table.

Approximate figures for the content of the samples in International Units, obtained by multiplying by 2.6 and rounding off the values to the nearest hundred, are given in column 2.

As the Table clearly shows, the liver oil of the shark, and of the related saw-fish, is a rich source of vitamin A. The samples of shark-liver oil showed considerable variation in potency, but only one, a sample of somewhat uncertain origin, had a vitamin A content below that of good cod-liver oil, which is in the neighbourhood of 1,000 International Units per gramme. The average value given by the 25 samples of shark-liver oil was about 10,000 International Units per gramme, and that of the saw-fish samples about 12,000.

Variation in vitamin A content is no doubt due to several factors species, sex, feeding habits, season, the method of collection and preparation, etc. The hammer-head sharks appear to supply the richest oils, but a larger number of samples would have to be investigated to establish this point.

TABLE

Vitamin A content of fish-liver oils

		Vitamin A, micrograms per gramme	Vitamin A, International Units per gramme (approx.)
(a) Sharks (1) Hammerheads (<i>Sphyrna</i> sp) —			
Sample No.	{ 1	12,650	32,900
	2	9,140	23,800
	3	7,380	19,200
	4	7,320	19,000
	5	5,620	14,600
	6	3,510	9,100
	7	3,510	9,100
	8	3,170	8,200
(2) Man-eating sharks (<i>Galeocerdo tigrinus</i>) —			
Sample No.	{ 1	5,400	14,000
	2	630	1,600
	3	520	1,400
	4	360	900

TABLE—concl'd

		Vitamin A, micrograms per gramme	Vitamin A, International Units per gramme (approx)
(3) Common shark (Carcharinus) —			
Sample No	1 (<i>Carcharinus gangeticus</i>)	10,100	26,300
	2 (<i>Carcharinus lemminckii</i>)	9 000	23,400
	3 'Large shark' (<i>Carcharinus gangeticus</i>)	3,750	9,800
	4	3,160	8,200
	5 (<i>Carcharinus melanopterus</i>)	3,160	8,200
	6 (" ")	3,000	7,800
	7 (" ")	1,980	5,100
	8	1,800	4 700
	8 (Stearine separated from this oil)	990	2,600
	9	980	2,500
	10	880	2,300
	11	60	200
12 (<i>Carcharinus melanopterus</i>)		Trace	
(b) Saw fish (Pristis) —			
Sample No	1	9,930	25,800
	2	3 940	10,200
	3	3,060	8,000
	4	1 870	4,900
(c) Rays —			
Sample No	1	250	700
	2	250	700
	3	210	600
	4	140	400
(d) Plough fish (<i>Rhynchobatus diadema</i>)		400	1,000
(e) Hilsa (<i>Hilsha ilisha</i>)		1	

The essential point is that shark- and saw-fish-liver oil is in general a most potent source of vitamin A. Liver oil collected in bulk from these fish, without regard to species, would be of great value in the treatment of vitamin A deficiency, which is extremely common in India. This is a question which has assumed considerable importance since the outbreak of war. Cod-liver oil has hitherto been generally used in India for the treatment of keratomalacia and other forms of vitamin A deficiency and has also been widely employed for improving the condition of malnourished children. It is obvious that cod-liver oil will not be obtainable from Europe as long as the war lasts. An opportunity is thereby provided for developing an indigenous industry for the production of fish-liver oils rich in vitamin A.

SUMMARY

1 The vitamin A content of 25 samples of shark-liver oil, and 4 samples of saw-fish-liver oil, has been determined. The average content was found to be about 10,000 and 12,000 International Units per gramme respectively.

2 The value of these oils as a substitute for cod-liver oil as a source of vitamin A is emphasized.

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DIET SURVEYS IN THE CENTRAL PROVINCES AND BERAR

BY

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[Received for publication, September 25, 1940]

THIS paper records the results of diet surveys in urban and rural areas in the Central Provinces, carried out during the months of November 1939 to April 1940. The following groups were investigated —

- (1) *Nagpur*—22 families employed in cotton mills
- (2) *Tirodi*—29 families employed in a manganese mine
- (3) *Warud* (a village some 20 miles from Nagpur)—20 families belonging to the poor cultivator class

An attempt was also made to assess the nutritional state of children in these places, some 5,000 children having been examined

Nagpur—The survey was carried out over a period of 15 days in December. The families belonged to the poorer classes of industrial workers, with monthly incomes ranging from Rs 10 to Rs 32 per family, with an average of Rs 18-8-0. The average number of persons per family was 5.5. Intake of various foods per consumption unit (c u) per day and the chemical composition of this average diet are shown in Table I —

TABLE I

Diet of mill-hands in Nagpur

Food	Oz per c u daily	Chemical composition	Per c u daily
Rice (raw, under milled)	16.5	Total protein	60 g
Wheat	2.5	Animal ,	3 g
Pulses	2.1	Total fat	23 g
Leafy vegetables	0.9	Animal ,,	7 g

TABLE I—*concl'd*

Food	Oz per c u daily	Chemical composition	Per c u daily
Non-leafy vegetables	3.2	Carbohydrate	472 g
Fruit	None	Calories	2,400
Ghee	0.14	Percentage of calories from cereals	76
Vegetable oil	0.51	Calcium	0.3 g
Milk and buttermilk	0.85	Phosphorus	1.3 g
Meat, fish and eggs	0.27	Vitamin A (International Units)	1,100
Sugar and jaggery	None		

The results of this survey confirm the findings of Aykroyd, Krishnan, Passmore and Sundararajan (1940) that the diet of the poor rice-eater is very much the same all over India. The composition of the diet consumed by the mill-hands in question closely resembles 'the cheap Madrassi diet' which has been used in numerous animal experiments in the Coonoor Laboratories. It is deficient in animal protein, animal fat, calcium, vitamin A and in certain constituents of the vitamin B₂ complex. If the standard of adequate calorie intake is set at 2,600 daily, 13 out of 22 families were underfed. A similar analysis in the case of calcium showed that intake exceeded 0.4 g daily in only 3 families. Sherman's standard of adequate calcium intake is 0.68 g. If highly-milled raw rice replaced under-milled raw rice, the diet would become a beriberi-producing one.

Tirodi—The survey lasted 18 days and was carried out in March. The average monthly income of the 29 families was estimated to be Rs. 16. The number of persons per family in this group was only 2.7, as compared with 5.5 in the Nagpur families. Average monthly income per consumption unit was Rs. 7-2-0 and Rs. 4-8-0 respectively. The difference in the number of persons per family in the two groups is of some social interest. The Nagpur workers, living in a large city, had to maintain numerous related dependants and in many cases there was only one wage earner per family. The industrial workers in Tirodi, on the other hand, having migrated from their original homes in search of employment, were much freer of dependants, and in many families both husband and wife were working. While total income per family was smaller in the Tirodi group, *per capita* income

was larger, and this was reflected in the fact that this group consumed more rice than the Nagpur mill-hand families

TABLE II

Diet of industrial workers in Tirola

Food	Oz per c u daily	Chemical composition	Per c u daily
Rice (home pounded, parboiled)	21.5	Total protein	78 g
Wheat	1.0	Animal „	2.7 g
Pulses	2.5	Total fat	28.1 g
Leafy vegetables	0.5	Animal „	2.8 g
Non leafy vegetables	4.8	Carbohydrate	581 g
Fruit	None	Calories	2,900 g
Ghee	0.3	Percentage of calories from cereals	78
Vegetable oil	0.6	Calcium	0.39 g
Milk and buttermilk	Negligible	Phosphorus	1.6 g
Meat, fish and eggs	0.5	Vitamin A (International Units)	1,400
Sugar and jaggery	None		

This again is a typical 'poor rice-eater's diet'. It differs from the diet shown in Table I mainly in its greater content of rice and higher calorie yield. Only 6 families (20 per cent) had a calorie intake below 2,600. Qualitatively the diet shows much the same defects as the Nagpur diet. In 17 families (59 per cent) intake of calcium was less than 0.4 g per c u daily.

Warud—This is a village containing some 600 families, situated $3\frac{1}{2}$ miles from the main road. The 20 families surveyed all belonged to the poor cultivating class and were typical of the vast majority of families living in the village. The survey was carried out for 16 days in January and February. It is of interest in that the staple food of the villagers is millet (cholam, *Sorghum vulgare*). Only one

other survey in a millet-eating area has hitherto been carried out in India—in a ragi-growing district in Closepet, Mysore (Aykroyd, 1940)

The average number of persons per family was 5.35. No attempt was made to estimate income.

The diet and its chemical composition are shown in Table III —

TABLE III
Diet of cultivators in Warud

Food	Oz per c u daily	Chemical composition	Per c u daily
Cholam	16.0	Total protein	68 g
Wheat	1.5	Animal „	1.1 g
Rice	0.1	Total fat	20.0 g
Pulses	2.0	Animal „	1.2 g
Leafy vegetables	Negligible	Carbohydrate	420 g
Non leafy vegetables	4.5	Calories	2,100
Fruit	None	Percentage of calories from cereals	82
Ghee	None	Calcium	0.43 g
Vegetable oils	0.31	Phosphorus	1.88 g
Milk and buttermilk	1.8	Vitamin A (International Units)	1,000
Sugar and jaggery	Negligible		

In composition this diet closely resembles the poor rice-eater's diet, except that cholam replaces rice. Cholam has a higher protein and calcium content than rice and intake of these factors was higher than in the case of the rice-eating groups in spite of the low calorie yield of the diet. All but 3 families were underfed on the 2,600 calorie standard. Apart from total protein and calcium content the diet shows much the same qualitative defects as the poor rice-eater's diet.

Examination of children—Height and weight records were obtained of 4,690 children (4,185 boys and 505 girls) in Nagpur. These were mostly children of the poorer classes attending schools in the null area. Averages are shown in Table IV —

TABLE IV

Average height and weight of children in Nagpur

Age	Number of boys	Number of girls	AVERAGE HEIGHT (INCHES)		AVERAGE WEIGHT (LB)	
			Boys	Girls	Boys	Girls
5	22		42.3		38.0	
6	320	24	42.5	44.8	36.6	38.9
7	792	96	45.0	44.7	36.4	43.7
8	796	108	46.6	46.7	42.4	45.5
9	762	96	46.7	48.8	46.8	49.8
10	579	91	50.2	50.0	49.7	56.8
11	390	36	52.0	52.0	54.2	59.7
12	284	19	53.4	56.3	56.3	70.0
13	159	12	54.9	58.0	63.9	76.5
14	80		57.1		74.9	
15	38		59.7		80.7	

Height and weight averages were approximately similar to those of children of the poorer classes in other rice-eating areas of India, suggesting that the growth rate of children between the ages of 5 and 15 may be largely determined by diet and that differences in race have relatively little influence on physical development in these age groups.

Deficiency diseases—The incidence of phrynoderma, xerophthalmia and stomatitis provides a useful index of the state of nutrition of children (Aykroyd

and Rajagopal, 1936) Of 4,185 children examined in Nagpur, 675 (16 per cent) showed one or more of these conditions In Tirodi, only 149 boys were examined, 46 per cent were found to be suffering from deficiency diseases The higher incidence in the Tirodi group is difficult to explain Possibly it is due to the fact that the large numbers examined in Nagpur included a larger proportion of children belonging to families of a better economic status than that of the 22 families surveyed The important point, however, is that the examinations revealed a very high incidence of deficiency disease in both groups In Warud and surrounding villages, 27 per cent of 107 children were found to be suffering from deficiency disease It is of interest to record that in the Nagpur group (boys) 84 per cent consumed no milk, while in the Warud group the percentage was 93

Twenty to twenty-five per cent of all the children examined had one or more teeth showing gross caries Altogether the picture obtained was that of a wretched state of nutrition among children in the Central Provinces It is noteworthy that this province has the highest death rate in British India

SUMMARY

1 Diet surveys have been carried out on families of industrial workers in Nagpur and Tirodi, Central Provinces, and in families in Warud village, Central Provinces

2 The diet of industrial workers was a typical 'poor rice-eater's diet' deficient in many respects The village diet was based on cholam (*Sorghum vulgare*) but otherwise similar in composition to the diets of the urban workers

3 A high incidence of deficiency diseases was observed among children in these places

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STUDIES IN HUMAN NUTRITION

Part III

PROTEIN, CALCIUM AND PHOSPHORUS METABOLISM WITH TYPICAL INDIAN DIETARIES

BY

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ALTHOUGH diet surveys yield valuable information about the adequacy of diets in particular areas, such surveys are always open to a certain amount of error. It is not always possible to make correct allowances for the portions of foodstuffs not actually eaten and there is some uncertainty with regard to accuracy of the scales of family coefficients used for determining intake per consumption unit. In India it has been assumed that adult requirements of proteins and minerals are the same as those of people in Europe and America. That this assumption appears to be justified has been shown by the recent investigations of Basu and Basak (1939) and of Basu, Basak and Rai Sircar (1939) in this laboratory.

In the present investigation we have studied the adequacy of some typical Indian diets with regard to protein, calcium and phosphorus by direct metabolic experiments on a number of individuals. The experimental subjects had to lead very disciplined lives, taking a uniform and weighed diet, which they consumed *in toto*, for a number of days. The analysis of the foodstuffs taken and of the urine and faeces revealed whether they were in positive or negative balance with regard to a particular element.

Experiments with nitrogen-free diets have also been performed to determine the endogenous nitrogen metabolism of Indians.

EXPERIMENTAL

The selection of diets which might be claimed as representative is difficult in a subcontinent like India where dietary habits and available foodstuffs are so different in different areas. Three 'typical' diets were chosen (1) a non-vegetarian rice diet containing fish, pulse and vegetables resembling that commonly taken by the people of Bengal and Assam and certain parts of the Deccan, (2) a vegetarian rice diet containing pulse and vegetables as consumed in Orissa, Madras, Mysore and other parts of Southern India and (3) a vegetarian diet based on whole wheat (atta) containing pulse and vegetables as eaten by the people of the U P, the Punjab and other parts of Western and Central India. The composition of the diets is shown in Table I. The diets were adequate in calorie content.

Experiments were conducted on five healthy human adults B C R S, G C N, S N D, U C S and P C D. Their ages varied between 19 and 28 years.

The experimental procedure was the same as that reported in two previous communications (Basu *et al*, *loc cit*). The experimental diets were taken for 6 or 9 days and immediately after this period 10 oz of cow's milk was added. The milk supplement was continued for 6 days. The analytical methods for the estimation of nitrogen, calcium and phosphorus were the same as employed in the previous investigations.

With a particular diet, the period of first 3 days was considered to be preliminary and the urine and faeces of each subsequent period of 3 days were collected and analysed and the mean of the daily analytical figures is presented.

RESULTS

The elimination of nitrogen on a protein-free diet is shown in Table II. The intake and output of nitrogen, calcium and phosphorus on typical diets are given in Tables III and IV. The biological value and digestibility of the mixed proteins of the diets are shown in Table V.

TABLE I

Composition of the diets

Foodstuffs	(1) Rice fish diet (g)	(2) Vegetarian rice diet (g)	(3) Whole wheat diet (g)
Rice	550-600	600	
Whole wheat (atta)			500-550
Pulse	60	100	70-90

TABLE I—*concl'd*

Foodstuffs	(1) Rice fish diet (g)	(2) Vegetarian rice diet (g)	(3) Whole wheat diet (g)
Fish	70		
Vegetables	200	200	200
Sugar			50
Mustard oil	30	30	
Ghee			30

(4) Nitrogen free diet (g)	
Sago	550 to 600
Sugar	200

TABLE II

Experiment with nitrogen-free diet.

Experimental subject	Urine volume (c c)	Urinary nitrogen per day (g)	Fæcal weight (dry) per day (g)	Fæcal nitrogen per day (g)	Total nitrogen output per day (g)
P C D	1,190	1 850	23 1	1 030	2 880
G C N	2,320	2 221	22 7	1 049	3 270

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Rice	550-600	600	
Whole wheat (atta)			500-550
Pulse	60	100	70-90

TABLE III—*cont'd*

Experimental subject	Experimental diet	Experimental period (P)	PROTEIN METABOLISM				CALCIUM METABOLISM				PHOSPHORUS METABOLISM			
			Dietary N per day (g)	Urinary N per day (g)	Faecal N per day (g)	Balance (g)	Dietary Ca per day (mg)	Urinary Ca per day (mg)	Faecal Ca per day (mg)	Balance (mg)	Dietary P per day (mg)	Urinary P per day (mg)	Faecal P per day (mg)	Balance (mg)
P C D	Rice fish diet	P 2	12 735 5 937	Protein intake—70 ± g	3 015 + 3 783		193	68	317	-192	1,344	666	552	+ 116
"	"	P 3	13 806 6 293	Protein intake—86 7 g	3 262 + 4 311		536	80	305	+ 61 1,645	(Dietary)—Ca/P=1/6 9	650	555	+ 440
"	"	P 4	8 520 5 034	Protein intake—86 7 g	3 350 + 0 136		221	20	218	-17 1,023	(Dietary)—Ca/P=1/3 1	507	451	+ 65
G C N	*Rice fish diet	P 2	8 520 4 912	Protein intake—53 2 g	3 164 + 0 344		221	28	223	-30 1,023	(Dietary)—Ca/P=1/4 6	476	421	+ 126
"	"	P 3	9 923 5 531	Protein intake—62 0 g	3 410 + 0 982		595	36	435	+ 124 1,312	(Dietary)—Ca/P=1/2 2	512	517	+ 283
"	*Rice fish diet and milk	P 4	12 56 5 576	Protein intake—78 5 g	3 483 + 3 501		200	17	194	-11 1,084	(Dietary)—Ca/P=1/8 4	479	935	+ 280
G C N	Rice diet	P 2	13 90 5 837	Protein intake—86 8 g	4 046 + 4 017		584	30	386	+ 168 1,978	(Dietary)—Ca/P=1/3 4	528	887	+ 563
"	"	P 4												

Each diet was taken for a number of days and then supplemented with 10 oz cow's milk (except in the case of B C R S when 16 oz milk was taken) and the supplementation continued for several days. Each period included 3 consecutive days and only the mean of the daily analytical figures for each period is presented. The chronological order of the experimental periods is denoted by arithmetical numbers and the periods which do not figure in the table must be assumed to be the preliminary periods for the succeeding ones.

* Rice used in these experiments was highly polished.

TABLE IV.

Metabolism on whole-wheat (atta) diet

Experimental subject	Experimental diet	Experimental period (P)	PROTEIN METABOLISM				CALCIUM METABOLISM				PHOSPHORUS METABOLISM			
			Dietary N per day (g)	Urinary N per day (g)	Faecal N per day (g)	Balance (g)	Dietary Ca per day (mg)	Urinary Ca per day (mg)	Faecal Ca per day (mg)	Balance (mg)	Dietary P per day (mg)	Urinary P per day (mg)	Faecal P per day (mg)	Balance (mg)
U C S	Whole wheat (atta) diet	P 2	12 118	7 686	1 912	+ 2 520	302	126	190	- 13	1,576	706	487	+ 383
"	"	P-3	12 118	8 445	1 728	+ 1 945	302	106	197	- 1	1,576	838	482	+ 256
			Protein intake—75 8 g				(Dietary)—Ca/P=1/5 2							
"	Whole wheat diet and milk	P 4	13 266	8 025	1 818	+ 3 423	676	108	336	+ 232	1,865	651	668	+ 546
"	"	P 5	13 266	8 352	1 762	+ 3 152	676	101	260	+ 306	1,865	612	496	+ 757
			Protein intake—82 9 g				(Dietary)—Ca/P=1/2 7							
G C N	Whole-wheat (atta) diet	P 2	14 279	6 970	2 364	+ 4 945	300	20	218	+ 62	1,875	751	621	+ 503
"	"	P 3	14 279	7 517	2 768	+ 3 994	300	23	223	+ 54	1,875	670	652	+ 553
			Protein intake—89 3 g				(Dietary)—Ca/P=1/6 2							
"	Whole-wheat diet and milk	P 4	15 451	7 910	2 847	+ 4 694	674	20	410	+ 264	2,164	782	786	+ 596
"	"	P 5	15 451	8 259	2 489	+ 4 703	674	17	380	+ 294	2,164	808	678	+ 618
			Protein intake—96 6 g				(Dietary)—Ca/P=1/3 2							

Each diet was taken for a number of days consecutively and then supplemented with 10 oz cow's milk and the supplementation continued for several days. Each period included 3 consecutive days and only the mean of the daily analytical figures for each period is presented. The chronological order of the experimental periods is denoted by arithmetical numbers and the periods which do not figure in the table must be assumed to be the preliminary periods for the succeeding ones.

TABLE V
Biological value and digestibility of the mixed proteins of the different diets

Experi- mental subject	RICE DIET				Experi- mental subject	WHOLE WHEAT (ATTA) DIET			
	Diet	Level of protein intake per day (g.)	Biological value, per cent	Digestibility, per cent		Diet	Level of protein intake per day (g.)	Biological value, per cent	Digestibility, per cent
P C D	Rice fish diet	70.0	02	85	G C N	Atta diet	80.3	03	91
"	Rice fish diet and milk	80.3	02	84	"	"	80.3	58	88
S N D	Rice fish diet	75.0	50	79	"	Atta diet and milk	90.0	58	88
"	Rice fish diet and milk	84.1	60	80	"	"	96.0	57	91
G O N	Rice diet	78.5	07	81					
"	Rice fish diet	53.2	55	73		Mean	93	59	89.5
"	"	53.2	58	75					
"	Rice fish diet and milk	02.0	50	70					
	Mean	73	60	80					

DISCUSSION

Protein

The minimum nitrogen expenditure—By this is meant the minimum amount of total nitrogen excreted in the urine and faeces by normal adults living on a protein-free diet of adequate calorie content. The elimination of nitrogen in the urine and faeces together under such conditions was 2.9 g in the case of P C D and 3.3 g in the case of G C N or 0.61 g and 0.65 g per kilo of body-weight respectively. Hence the smallest amount of protein theoretically necessary to make good endogenous losses was 18.0 g in the case of P C D and 20.4 g in the case of G C N with a mean value of 19.2 g per man value, or 26 g per adult of 70 kilo of body-weight. This value is exactly the same as that estimated from the results of the previous experiments (Basu and Basak, *loc cit*).

Protein nutrition on typical diets—The protein content of the diets varied between 76 g in the case of the rice-fish diet and 82 g in the case of the vegetarian whole-wheat (atta) diet, while that of one rice-fish diet (Table III) containing highly polished rice was only 53 g. The addition of milk (10 oz) to those diets increased protein content by about 8 g.

Not only were the subjects in positive nitrogen balance in all the experiments but they retained considerable amounts of protein in their bodies. The average retention of protein on the rice-fish diet (containing 76 g protein) was about 13 g. On both the whole-wheat (atta) and vegetarian rice diets with a protein intake of 78 g to 82 g the average retention of protein was about 22 g. The addition of 10 oz cow's milk, which increased the protein contents of the diets from 86 g to 90 g augmented the protein retention of the subjects by about 5 g and, consequently, the total protein gained by the subjects on the milk-containing diets was 18 g to 27 g.

Although the minimum protein requirements of human adults for maintenance have been determined, nevertheless human requirements for the maintenance of perfect health and activity have not yet been defined with any degree of accuracy. For practical purposes the minimum protein requirement for maintenance together with an extra allowance of 50 per cent may tentatively be accepted as a safe figure. From the results of laboratory researches and dietary surveys, Sherman (1920a) calculated the minimum protein requirement for maintenance to be 44.4 g and, adding an extra 50 per cent to this value, set the standard at 66.6 g. Sherman recommended a standard allowance of 1 g of protein per kilo of body-weight per day and he claimed that such an allowance will provide a margin of safety of 50 to 100 per cent as far as adult requirements are concerned. The Technical Commission of the Health Committee of the League of Nations (1936) recommended the same amount of protein, namely, 1 g protein per kilo of body-weight for an adult, although it recommended 105 g of protein for pregnant and nursing mothers. Stiebeling (1933) compiled a set of standards based on the relative requirements of children, adolescents and adults, male and female, in which the average protein requirement per head of the population was calculated to be 68 g. Orr (1937) accepted the Stiebeling standard in his survey of diet in the British Isles. Basu

and Basak (*loc cit*) determined the minimum maintenance protein requirement of Indian adult as 46.4 g per 70 kilo of body-weight. If a 50 per cent margin of safety is allowed for, the protein requirement of an adult weighing 70 kilo will be 69.6 g. The latter value is, therefore, very similar to that put forward by both Sherman (1920a) and Stiebeling (*loc cit*). If our present results are compared with these standards it is clear that the diets, even without milk, are quite adequate in protein content and can satisfy all the accepted standards except that of the British Medical Association (1933) which is generally regarded as too high. On the addition of milk the protein content of the diets surpassed all the accepted standards and approached even that of the British Medical Association. Typical Indian dietaries are, therefore, adequate in protein content and permit satisfactory utilization of protein, although they do not, in general, contain much protein of animal origin. The contention of McCay (1912) and of Wilson that 'the protein element in nutrition in India may ultimately prove to be the most important and it will certainly be the most difficult to remedy' (Wilson, Ahmad and Mullick, 1936) and 'the main deficiency is one of protein primarily' (Wilson, Ahmad and Mitra, 1937) cannot be sustained provided the diets contain sufficient amounts of pulse and/or of fish. Actually one of the most important deficiencies of Indian diets is not of protein but of calcium—a deficiency which is as serious as it is difficult to remedy in view of economic and other considerations.

Biological value and digestibility of the mixed proteins of mixed diets—Although the determination of the biological value and digestibility of the proteins of individual foodstuffs is a valuable guide in their selection in the daily menu, nevertheless, knowledge of the corresponding values of the mixed proteins of actual dietaries is more important.

Basu and Basak (*loc cit*) found the biological values and digestibilities of the proteins of rice diets to be 75 and 62 respectively at a protein level of 40 g and those of wheat diets to be 66.5 and 78, at a protein intake of 52 g. This showed that the proteins in rice diets were more effectively utilized but were less digestible than those of wheat diets. In this investigation the corresponding values for the mixed proteins of rice diet were found to be 60 and 80 at the much higher level of protein intake of 73 g, while those for proteins of wheat diet were 59 and 89.5 respectively at a level of protein intake of 93 g. Thus, the biological values of mixed proteins of both rice and wheat diets were found to be very nearly similar. Although the biological value of the proteins of rice and wheat diets somewhat decreased as might be expected, at the higher levels of protein intake, the digestibility coefficient increased considerably, the increase being 18 per cent in case of the rice diets and 11.5 per cent in the case of wheat diets. Increase in the level of intake thus appears to improve the digestibility of the proteins in mixed diets.

Calcium

Calcium requirement—The maintenance requirement of calcium or phosphorus for adults is generally estimated from the total amount of the element in question excreted by the subjects when just in balance, and standards for purposes of

practical nutrition are based on the maintenance requirement [i.e. the minimum requirements for the maintenance of a positive balance in laboratory studies (Orr, *loc cit*)], plus a 50 per cent allowance for the additional requirements of everyday life and the maintenance of health and activity. In extensive studies including 97 experiments, Sherman (1920b) found daily calcium outputs which ranged from 0.27 g to 0.82 g with an average value of 0.45 g per 70 kilo of body-weight. Allowing for a 50 per cent safety margin, the dietary standard became 0.68 g. Stäbeling (*loc cit*), however, set the standard at 0.90 g representing the average requirement of a family including children, adolescents and adults, male and female. On the other hand, Leitch (1937), applying another method of calculation to about 400 balance experiments on non-pregnant non-lactating women, determined the maintenance requirement to be 0.55 g daily per woman value (not per 70 kilo of body-weight), while Owen (1939) in his studies of the calcium requirement of older subjects, who are more prone to senile osteoporosis supposed to be caused mainly by long-standing calcium deficiency, determined the requirement value of older males as 0.52 g. Basu *et al* (*loc cit*) calculated the maintenance calcium requirement to be 0.388 g per 70 kilo of body-weight. This was based on metabolic experiments on only three subjects, the content of calcium in the diet was small and insufficient, and the calcium metabolism of one of the subjects (G C N) was found to be abnormally low. The present investigations (*vide tables*) are based on a large number of experimental subjects living on diets of higher calcium content and allowance has been made for the fact that calcium requirements 'show no correlation with weight' (Leitch, *loc cit*) and that 'the calcium requirements of older males are much the same as those of the younger adults' (Owen, *loc cit*). The conversion of the calcium requirement into terms of requirements per 70 kilo of body-weight is not, therefore, justified. In Table VI the values obtained for the calcium requirement of the different experimental subjects have been collected together.

From the table it would appear that the maintenance calcium requirement is 0.432 g per man value. Accepting this value and adding a 50 per cent allowance it is found that 0.648 g calcium per day is the requirement of an adult for maintaining health and activity.

Calcium metabolism on typical dietaries and the effect of a milk supplement — The calcium contents of the rice-fish diets were found to be 193 mg to 280 mg with a mean value of 236 mg and that of the vegetarian rice diet was 200 mg, whereas the vegetarian atta diets contained about 300 mg calcium on an average. If the calcium contents of these diets are compared with the human calcium requirement as discussed above, it will be clear that these dietaries are deficient in calcium content and fail to meet even the bare maintenance requirement.

In almost all experiments with the rice and the rice-fish diets, the subjects were in negative calcium balance and often the total calcium outputs were such as to exceed the intake by about 192 mg (as in subjects U C S and P C D). The addition of 10 oz of cow's milk increased the calcium intakes of these two subjects to 578 mg and 536 mg respectively, at these levels of intake one subject just maintained balance, while the other retained 69 mg of calcium. Although both G C N (with an abnormally low calcium requirement) and B C R S were

approaching balance on rice diets alone, and retained fair amounts of calcium when milk was added to such diets, nevertheless it was striking that the deficit calcium budget of the subject S N D could not be balanced even on the addition of 10 oz milk.

TABLE VI

The calcium requirement of Indians

Experimental subjects	Body weight (kilo)	Number of experiments	CALCIUM REQUIREMENT FOR MAINTENANCE		MINIMUM CALCIUM REQUIREMENT FOR JUST MAINTENANCE	
			Per man value (g)	Per 70 kilo of body weight (g)	Per man value (g)	Per 70 kilo of body weight (g)
G C N	49 (19 years)	23	0 174	0 248	0 158	0 226
B C R S	54 (22 years)	7	0 423	0 548	0 232	0 301
S N D	49 (24 years)	7	0 407	0 581	0 407	0 581
G C N	50 (20 years)	10	0 322	0 451	0 234	0 327
U C S	47 (28 years)	7	0 426	0 634	0 338	0 503
S N D	49 (25 years)	3	0 567	0 810	0 361	0 516
P C D	45 (22 years)	3	0 451	0 701	0 385	0 599
Average for last six subjects		36	0 432	0 621	0 326	0 471
Average for all subjects		59	0 396	0 568	0 302	0 436

Similarly, on vegetarian atta diets containing 302 mg calcium, U C S lived in negative calcium balance, although the deficit was small and not so striking as in the experiments with rice diets. In calcium content wheat diets are better than rice diets either vegetarian or non-vegetarian. The addition of 10 oz milk increased the total calcium content of the diet to 676 mg, and resulted simultaneously in a favourable retention of 269 mg. G C N was almost in balance on the atta diet and retained 279 mg calcium when milk was added.

Aykroyd and Krishnan (1937) came to similar conclusions as a result of their growth experiments with rats. Young rats on a poor rice diet showed very poor growth. Almost normal growth was obtained when a supplement of calcium lactate or skimmed milk powder was added but not on the addition of casein. The same workers (1938) showed that the growth rate of nursery school children living on a rice diet could be enhanced by the regular administration of calcium lactate.

The two most important points that emerge from our investigations are that (1) Indian dietaries (without milk) do not satisfy calcium requirements and (2) that milk is of great value in making good this deficiency. The amount of milk (10 oz) used in the experiments covered the maintenance requirements of calcium but did not always provide a 50 per cent margin of safety.

Even this small amount of milk, however, is beyond the means of most of the Indians of the poorer classes. In order to ensure an adequate intake of calcium by millions of poor Indians it is essential to find out some very cheap and at the same time rich sources of nutritionally available food calcium. The availability of vegetable calcium as also that of calcium from other sources is now under investigation in this laboratory.

Phosphorus

In a previous investigation (Basu *et al*, *loc cit*) the human phosphorus requirement was found to be 1.001 g per 70 kilo of body-weight, a figure in concordance with the estimates of Sherman (1920c) (0.88 g) and Owen (*loc cit*) (1.20 g). The dietaries used in the present experiment contained from 1,334 mg to 1,875 mg phosphorus with an average of 1,623 mg. They provided not only the maintenance phosphorus requirement but also a 50 per cent margin of safety. The mean phosphorus content of the diets supplemented by 10 oz of milk was 1,915 mg, allowing a 100 per cent margin of safety. All the subjects were in positive phosphorus balance and on an average retained 397 mg. On the addition of milk, there was a greater retention of phosphorus, namely 507 mg. So far as the phosphorus content is concerned, Indian dietaries are perfectly adequate and satisfactory.

SUMMARY

1. Nitrogen, calcium and phosphorus metabolism experiments have been performed on human subjects consuming typical Indian diets.

2. The minimum nitrogen expenditure of two human subjects, fed on a protein-free diet of adequate energy value, was 18.0 g and 20.4 g with a mean value of 19.0 g per man value or 26 g per adult of 70 kilo of body-weight.

3. The protein contents of a rice and fish and a vegetarian whole-wheat (atta) diet varied between 76 g and 82 g and on these diets the subjects were in positive nitrogen balance and retained considerable amounts of protein.

4. The biological values and digestibilities of the proteins of mixed rice and of mixed wheat diets were 60, 80 (rice), and 59 and 89.5 (wheat), respectively.

5 From the average of 36 metabolism experiments conducted on five experimental subjects at different times, the maintenance calcium requirement was calculated to be 0.432 g per man per day

6 The calcium content of typical diets and the capacity of these diets to supply the calcium requirements of subjects have been determined. The diets were too low in calcium, the rice-fish diet containing on average 234 mg and the wheat diet about 300 mg of calcium. On these diets the experimental subjects were almost always in negative calcium balance. *One of the greatest defects of typical Indian dietaries is insufficiency of calcium*

7 The addition of 10 oz of cow's milk per day increased the calcium content of the diets (536 mg to 676 mg) and improved the calcium retention of the subjects considerably

8 Indian dietaries were found to be adequate with regard to their phosphorus content

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NUTRITION AND ITS BEARING ON PREVENTABLE BLINDNESS AND EYE DISEASES IN BENGAL

PRELIMINARY REPORT

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THIS investigation was carried out to determine the role of nutrition, and particularly that played by vitamin A deficiency, in relation to preventable eye diseases and blindness in Bengal

Bose (1939) had previously investigated the vitamin A level in school and college students in Bengal. Taking a dial reading of 70 and above on Jean's biophotometer as an indication of normal vitamin A content of the system a normal level was found in 61 out of 65 examined. Similar observations on jail prisoners were undertaken in collaboration with Sankaran (1938). Of 600 prisoners examined 500 belonged to the lower division and 100 to the higher division. The main difference was that a better class diet was given to the latter group. Of the lower division group 43 per cent showed a high degree of vitamin A deficiency and in the

higher division group the figure was 17 per cent. If dryness of the conjunctiva and Bitot's spots were taken as an index of vitamin A deficiency only 16·8 per cent of the lower class and 15 per cent of the higher class could have been diagnosed clinically.

For the purpose of study of the rôle of vitamin A deficiency in relation to eye diseases the ocular manifestations of vitamin A deficiency have been classified as follows —

- GROUP I — Hemeralopia without change in the fundus, cornea, etc
 GROUP II — Loss of lustre and presence of pigmentation in the conjunctiva
 Wrinkling of the bulbar conjunctiva with xerosis and Bitot's spots
 GROUP III — Diminished sensibility of the cornea with or without any other manifestations in the conjunctiva
 GROUP IV. — Keratomalacia proper without breaking of the cornea (loss of lustre and infiltration of the deeper layer of the cornea)
 GROUP V — Keratomalacia with breaking down of the cornea (corneal ulcer)
 GROUP VI — Phthisis bulbi or anterior staphyloma (absence of infection), or panophthalmitis (presence of infection)

OBSERVATIONS ON PATIENTS AT THE EYE INFIRMARY, MEDICAL COLLEGE, CALCUTTA

Out of 14,698 patients examined between 4th March and 25th June, 1940, 419 (2·85 per cent) have shown ocular conditions which can be attributed to vitamin A deficiency

Group	I	41	cases	Group	IV	28	cases
„	II	298	„	„	V	19	„
„	III	23	„	„	VI	10	„

The age incidence of cases was investigated as previous observers in Bengal had found that the commonest cause of blindness in children below the age of 5 years was keratomalacia. The condition had been found to be rare in adult Bengalees.

The following was the incidence of cases observed —

			Per cent
Up to 5 years	61	cases, keratomalacia	41 (71·92)
6 to 10 „	34	„ „	6 (10·52)
11 to 15 „	31	„ „	1 (1·75)
16 to 20 „	70	„ „	3 (5·24)
21 and upwards	223	„ „	6 (10·52)

(One due to obstructive jaundice of long duration, 5 due to poor diet and unhygienic condition of which 3 were non-Bengalees)

In the course of the investigations it was ascertained that diarrhoea, colitis, jaundice or phrynoderma had preceded the ocular affections, the incidence being as follows diarrhoea, 26 cases, colitis, 3 cases, jaundice, 5 cases, phrynoderma, 6 cases

The presence of Bitot's spots or pigmentation of the conjunctiva have been considered to be possible indications of vitamin A deficiency This point was investigated with the results shown in Tables I and II Out of 99 cases with pigmentation of the bulbar conjunctiva examined by the biophotometer 71 cases (71.7 per cent) showed normal vitamin A content Of 17 cases showing both pigmentation and Bitot's spots 15 (88.2 per cent) gave readings within the normal range These changes would not appear to be definite indications of vitamin A deficiency and may occur in its absence

TABLE I

Pigmentation of bulbar conjunctiva and biophotometer test (99 cases)

Biophotometer reading	Pigmentation of bulbar conjunctiva				TOTAL	
	+++	++	+	±		
90	0	2	0	1	3	71 cases (ie 71.71 per cent) showing no deficiency of vitamin A
85	0	4	2	3	9	
80	2	9	8	6	25	
75	2	5	3	0	10	
70	2	12	6	4	24	
65	3	2	1	0	6	28 cases (ie 28.28 per cent) showing deficiency of vitamin A
60	0	4	1	4	9	
55	1	1	0	0	2	
50	0	1	2	0	3	
45	0	1	0	0	1	
40	0	2	0	0	2	
35	0	0	0	0	0	
30	1	0	0	0	1	
20	0	0	1	0	1	
15	1	1	0	0	2	
10 with bright light	1	0	0	0	0	

TABLE II

*Bitot's spot with pigmentation of bulbar conjunctiva and biophotometer test
(17 cases)*

Biophotometer reading	Bitot's spot				TOTAL	
	+++	++	+	±		
90	0	0	0	0	0	15 cases (ie 88.23 per cent) showing no deficiency of vitamin A
85	0	1	1	0	2	
80	0	2	4	0	0	
75	0	0	0	0	0	
70	0	5	2	0	7	
65	0	0	0	0	0	2 cases (ie 11.76 per cent) showing deficiency of vitamin A
60	0	0	1	0	1	
55	0	0	0	0	0	
50	0	0	0	0	0	
10 with bright light	1	0	0	0	1	

TREATMENT OF EYE CONDITIONS DUE TO VITAMIN A DEFICIENCY

The administration of vitamin A preparations was tried in cases classified according to three categories —

First category

Clinical groups I, II and III

Second category

Clinical groups IV and V

Third category

Clinical group VI.

(1) Cases of first category were treated by oral administration of cod-liver oil or halibut-liver oil. If diarrhoea was present cod-liver oil was given by intunction until diarrhoea ceased after which oral administration was continued. Eight cases reported the results of treatment and these have been satisfactory. Two cases which did not improve rapidly were treated by injections of Prepahn (Glaxo Laboratories—100,000 I U vitamin A).

(2) Cases of the second category were treated by immediate injections of Prepahn. Of 17 cases treated the results were noted in 12.

(a) 5 cases—cornea cleared up completely after two injections at intervals of one week.

(b) 7 cases—cornea cleared up partially after one injection but they did not report after the second injection.

In this group 9 cases were treated with halibut-liver oil with the following results —

	Cases
Marked improvement	1
Improved	1
Improving	4
Not reported	3

Thirty-four per cent of the cases were suffering from diarrhoea

Excellent results were obtained in 2 cases of keratomalacia due to obstructive jaundice —

1st case—S H, 15 years, male, Mohammedan, schoolboy Keratomalacia secondary to obstructive jaundice Blood tested for van den Bergh Immediate direct positive

On admission—

R. E vision—No Fm Cornea—perforated and prolapsed iris

L E vision—Fm only Cornea—hazy and a small ulcer near lower limbus, sensibility diminished

Result after three injections of Prepalin (100,000 units of vitamin A in each dose)—

R E vision—Fm only Ulcer healed, adherent leucoma formed

L E vision—6/9 Cornea—completely cleared up except a small nebula at the site of the ulcer, sensibility restored

2nd case—F B, 42 years, female Mohammedan Keratomalacia affecting both the eyes following obstructive jaundice Blood tested for van den Bergh Immediate direct positive

On admission—

R E vision—Fc at 1 ft Cornea—hazy and diminished sensibility and a sloughing ulcer at the lower part of cornea affecting the pupillary area partially

L E vision—Fm only Cornea—as in R E

Result after four injections of Prepalin—

R E vision—6/24 Cornea—cleared up, ulcer healed up with the formation of leucoma Sensibility restored

L E vision—6/36 Cornea—clear and ulcer healed with formation of leucoma Sensibility restored

(3) Cases of third category

In this category all the patients were already blind But treatment was carried on for the improvement of the general health All the 10 cases showed marked improvement in general health in 3 to 4 weeks

SUMMARY

1 Nearly 3 per cent of patients attending the Eye Infirmary, Medical College, Calcutta, were found to be suffering from eye conditions due to vitamin A deficiency

2 Keratomalacia in children up to 5 years of age forms 71.9 per cent of all keratomalacia cases Vitamin A deficiency plays an important part

3 Conjunctival pigmentation with or without Bitot's spots is not necessarily due to vitamin A deficiency. A normal vitamin A content was found in 71.7 per cent of such cases as indicated by biophotometer examination.

4 Parenteral administration of vitamin A in cases of keratomalacia is very effective in curing the condition.

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AN ADSORPTION METHOD FOR THE ESTIMATION OF NICOTINIC ACID CONTENT OF FOODSTUFFS

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EVER since the effect of nicotinic acid and nicotinamide in the treatment of pellagra in man became established (Elvehjem, Madden, Strong and Wolley, 1937) attempts have been made to devise methods for the estimation of these constituents in foodstuffs. Although a number of methods have been suggested for their estimation in tissues, urine and blood (Karrer and Keller, 1938, Kingstad and Naess, 1938, Harris and Raymond, 1939, Bandier, 1939, Swaminathan, 1939, Ritsert, 1939, Vilter, Spies and Mathews, 1938, Pearson, 1939) very few methods are available for their estimation in foodstuffs.

Bandier and Hald (1939) have suggested a method for the estimation of nicotinic acid in yeast. Swaminathan (1938) has reported a method which involves (1) repeated extraction of the material with water, (2) treatment with lead acetate to precipitate proteins and their derivatives, (3) removal of lead by treatment with sulphuric acid, (4) evaporation to a small bulk, (5) hydrolysis with HCl to convert the nicotinamide into nicotinic acid, (6) removal of colour by boiling with charcoal and (7) estimation of nicotinic acid in the extract by CNBr reaction using aniline. A similar method has been suggested by Kingstad and Naess (1939) for the estimation of nicotinic acid and its amide in foodstuffs.

The methods so far suggested involve many steps and lack simplicity. It may be remarked that the charcoal which is used for removing the colour, adsorbs some nicotinic acid, which may vitiate the final result. Further, it was found by Bandier and Hald (*loc cit*) that lead acetate interferes with the colour reaction. Considering these defects and the time involved in carrying out the various steps, any simplification of the procedure would greatly increase the value of the method. It was with a clear recognition of these defects in the execution of the

methods that a new method based on adsorption and elution of nicotinic acid has been devised by which the estimations can be carried out in a much shorter time

No attempts have so far been made to apply adsorption methods for the estimation of nicotinic acid in foodstuffs. Adsorption methods are useful in removing materials which interfere with colour formation. Such methods are very elegant and specific. From a large number of adsorbents tried during the present investigation, the best results were obtained with medicinal charcoal (Merck). This charcoal is a very efficient adsorbent for nicotinic acid. The elution of the acid from the adsorbent can easily be made quantitative by using alcohol-sodium hydroxide mixture.

EXPERIMENTAL

Estimation of nicotinic acid—Of all the aromatic bases suggested for the colour reaction with nicotinic acid, the reagent *p*-aminoacetophenone, suggested by Harris and Raymond (*loc cit*), was found to be the best. The intensity of the colour obtained with this reagent is much higher than that given by metol (Bandier, *loc cit*) or aniline. In the present investigation the Harris and Raymond method was adopted for the colorimetric estimation of nicotinic acid.

A series of preliminary investigations was carried out to find the optimum conditions for the adsorption and elution of nicotinic acid from pure solutions of the vitamin.

A Adsorption

1 *Adsorption of nicotinic acid by various types of adsorbents*—Various types of charcoals, Fuller's earth and Permutit were examined for their adsorbing properties. Solutions containing 10 ml of M/15 phosphate buffer (pH 7.1), 10 ml water and 1,000 μ g nicotinic acid (B. D. H.) were taken in conical flasks, and 0.100 g of the adsorbent was added into each of the solutions. After 4 to 5 minutes' shaking, the charcoal was removed by centrifuging and the nicotinic acid in the solution was estimated after suitable dilution and adjustment of the pH of the solutions to 6.0. The results are set out in Table I—

TABLE I

Adsorption of nicotinic acid by various adsorbents

Adsorbent	Nicotinic acid originally present in the solution, μ g	Nicotinic acid in the residual solution after adsorption, μ g	Adsorption, per cent
Medicinal charcoal (Merck)	1,000	132	86.8
Blood charcoal	1,000	198	80.2
Norite charcoal	1,000	326	67.4

TABLE I—*concl'd*

Absorbent	Nicotinic acid originally present in the solution, μg	Nicotinic acid in the residual solution after adsorption μg	Adsorption, per cent
Decolorizing charcoal	1 000	625	37.5
Permutit	1,000	518	48.2
Fuller's earth	1,000	1 000	0

The results show that medicinal charcoal possesses the best adsorbing properties. In all subsequent investigations medicinal charcoal was used.

2 *Influence of pH on the adsorption of nicotinic acid by medicinal charcoal* — Adsorption was carried out as described above, in the presence of M/15 phosphate and M/5 acetate buffers of varying pH. The results are presented in Table II —

TABLE II

Influence of pH on the adsorption of nicotinic acid

pH	Nicotinic acid originally present in the solution, μg	Nicotinic acid in the residual solution after adsorption, μg	Adsorption, per cent
3.0	1,000	0	100
4.0		0	100
5.0		0	100
5.6	1,000	0	100
6.2	1,000	Trace	100
7.1	1,000	121.2	83
8.0	1 000	148.2	85

The results show that adsorption is more efficient in the acid than in the alkaline region. Thus acidity is favourable for adsorption on charcoal.

B Elution

For eluting nicotinic acid from the charcoal after adsorption, sodium hydroxide and alcohol were tried separately and mixed together in suitable proportions. After

various trials it was found that alcohol-NaOH mixture containing N/5 NaOH was better than either alcohol or sodium hydroxide alone. The elution was carried out by adding the alcohol-NaOH mixture to the adsorbate in 10 ml centrifuge tubes, and keeping these in a boiling water-bath for about 2 to 3 minutes with constant stirring with a glass-rod. After centrifugation the nicotinic acid was estimated in the eluate. The results are shown in Table III —

TABLE III

Elution of nicotinic acid from charcoal adsorbate

Elutrient	Nicotinic acid content of the charcoal adsorbate, $\mu\gamma$	Nicotinic acid in the eluate, $\mu\gamma$	Nicotinic acid eluted, per cent
10 ml absolute alcohol	1,000	517	51.7
8 ml absolute alcohol + 2 ml N NaOH (made up to 10 ml)	950	790	83.2
10 ml N/5 NaOH	880	170	19.2

By repeating the elution for a second time, the nicotinic acid can be completely and quantitatively recovered. Further, the elutrient was found to be best suited for medicinal charcoal, complete and quantitative elution not being achieved in the case of other charcoal adsorbents used. Table IV indicates the results obtained on the elution of nicotinic acid from the various charcoal adsorbents. The acid was eluted twice by means of alcohol-NaOH mixture using 10 ml of the elutrient each time.

TABLE IV.

Elution of nicotinic acid from the charcoal adsorbates by alcohol-NaOH

Adsorbates	Nicotinic acid in the adsorbate, $\mu\gamma$	Nicotinic acid in the eluate, $\mu\gamma$	Nicotinic acid eluted, per cent
Medicinal charcoal	868	847	97
Blood charcoal	802	573	72
Norite charcoal	674	512	76
Decolorizing charcoal	375	242	64

C *Principle of the method for the estimation of the nicotinic acid content of foodstuffs*

The method finally adopted was as follows —

(1) Extraction with water, (2) hydrolysis with NaOH to convert the nicotinamide into nicotinic acid (3) adsorption upon medicinal charcoal and elution in alcohol-NaOH solution, and (4) estimation of nicotinic acid by the cyanogen *p*-aminoacetophenone method of Harris and Raymond (*loc cit*)

(1) *Extraction* —For the extraction of nicotinic acid a 5-gramme sample of the material to be assayed is weighed into a flask, and extracted with water by keeping in boiling water-bath for 20 to 30 minutes, centrifuged, and the supernatant liquid decanted into a 100-ml flask. The extraction was repeated until the volume of the extract amounted to 100 ml. In the case of yeast, which is a rich source of nicotinic acid, 2 g were taken for extraction.

(2) *Hydrolysis* —For hydrolysis 25 ml or 50 ml of the extract, the amount depending on the concentration of nicotinic acid in the extract, were taken in conical flasks and 5 ml of 20 per cent NaOH were added for every 25 ml of the extract, and kept in a boiling water-bath for 20 to 30 minutes. Any precipitate formed was removed by centrifugation.

(3) *Adsorption and elution* —After hydrolysis the solution was brought to pH 6.0 to 6.5 (Bromothymol blue as external indicator) by adding concentrated HCl, and the precipitate formed was removed by centrifugation. To the clear extract thus obtained, which may be coloured yellow in some cases, 0.200 g of medicinal charcoal was added, and allowed to stand for 3 to 5 minutes with frequent shaking, and centrifuged in 50-ml centrifuge tubes. The adsorption was again repeated with 0.200 g of the charcoal. With some test substances, particularly yeast, traces of carbon are left floating on the top after centrifugation. The amounts of carbon present are, however, too small to affect appreciably the final result.

The combined adsorbates contained in the centrifuge tubes were eluted twice with alcohol-NaOH mixture* (10 ml of the elutrient being used each time), by keeping in a boiling water-bath for 2 to 3 minutes, till it begins to boil briskly, while stirring the charcoal vigorously with a glass-rod. After centrifugation (in 10-ml centrifuge tubes) the combined clear solution, which may be coloured yellow in certain cases, was neutralized to pH 6.0 or 5.6 after the addition of 1 ml of 4 per cent sodium bicarbonate solution, and made up to 25 ml or 50 ml with water. After neutralization the colour of the solution becomes less intense and in many cases the amount of colour is negligible.

(4) *Estimation of nicotinic acid in the elutrient* —The nicotinic acid was estimated by the cyanogen-*p*-aminoacetophenone method of Harris and Raymond. Standard solutions of nicotinic acid for colorimetric comparison were prepared by adding known amounts of the acid to the alcohol-NaOH mixture and adjusting the pH to that of the experimental solutions. As the eluate is sometimes coloured yellow,

* The alcohol NaOH mixture was prepared by mixing 20 ml of N NaOH and 80 ml of absolute alcohol and made up to 100 ml. It should be prepared fresh when required. The slight turbidity of the mixture can be removed by centrifugation.

due to the extraction of certain pigments, it is essential to include a blank value for the original colour of the solution. But in almost all the materials investigated, the colour of the original solution was so little compared to the intensity of colour due to the nicotinic acid present in the sample, that the blank value was not determined. In the case of maize, and pulses, however, which have a low nicotinic acid content the blank value (without the addition of CNBr) was included. The quantities of the reagents added for the development of the colour were the same as those suggested by Harris and Raymond, except that 0.2 ml of 10 per cent HCl was used for the development of the latent colour. In some cases it was found that after the addition of HCl, there was a slight change in the tint between the blank standard and the experimental solutions. This was probably due to change in pH. The addition of M/5 acetate buffer pH 5.6 (5 ml of buffer + 5 ml of the solution) to the solutions eliminated such changes in the tint and facilitated easy and accurate comparison of the colours in the colorimeter.

The method was applied to the determination of the nicotinic acid content of yeast, liver, cereals and pulses, and good recoveries of added nicotinic acid were obtained. The results are presented in Table V.—

TABLE V

Nicotinic acid content of foodstuffs with the percentage recovery of added nicotinic acid

Foodstuff	Amount of material used for analysis, g	Amount of nicotinic acid added, μ g	Amount of nicotinic acid found, μ g	Recovery of nicotinic acid, per cent	Nicotinic acid content, mg/100 g
1 Yeast	2.0		1,212		60.6
	2.0	300	1,500	94	
2 Rice (hand-pounded)	5.0		222		4.4
	5.0	100	320	98	
Rice (milled)	5.0		160		3.2
	5.0	100	250	90	
Rice (another variety)	5.0		107		2.1
	5.0		150		3.1
3 Wheat	5.0	100	235	85	
	5.0		80		1.6
4 Ragi (<i>Eleusine coracana</i>)	5.0	100	163	83	
	5.0		111		2.2
5 Cholam (<i>Sorghum vulgare</i>)	5.0	100	209	98	
	5.0				

TABLE V—*concl'd*

Foodstuff	Amount of material used for analysis, g	Amount of nicotinic acid added, μ g	Amount of nicotinic acid found, μ g	Recovery of nicotinic acid, per cent	Nicotinic acid content, mg/100 g
6 Cambu (<i>Pennisetum typhordeum</i>)	{ 5.0 5.0	100	160 246	86	3.2
7 Italian millet (<i>Panicum Italicum</i>)	{ 5.0 5.0	100	Trace 101	101	Trace
8 Maize I (<i>Zea mays</i>)	{ 5.0 5.0	200	9 94	43	<0.2
Maize II	5.0		29		<0.6
9 Black gram (<i>Phaseolus mungo</i>)	{ 5.0 5.0	100	115 104	79	2.3
10 Green gram (<i>Phaseolus radiatus</i>)	{ 5.0 5.0	100	18 106	88	<0.4
11 Red gram (<i>Cajanus indicus</i>)	{ 5.0 5.0	100	121 229	108	2.4
12 Bengal gram (<i>Cicer arietinum</i>)	{ 5.0 5.0	100	133 225	92	2.7
13 Sheep's liver	{ 5.0 5.0	100	506 586	80	10.1

DISCUSSION

The method is fairly quickly performed. It is designed so that the various steps adopted by other workers for the removal of proteins, etc. from the extract, are avoided. The recovery of added nicotinic acid in the case of maize was not as satisfactory as in the case of other cereals and pulses. Low recoveries of added nicotinic acid were repeatedly observed in the case of maize.

Since this work was completed a paper by Kodicek (1940) has appeared from the Nutritional Laboratory, University of Cambridge, in which the method of Harris and Raymond was applied to the estimation of nicotinic acid in animal tissues, blood and certain foodstuffs. Except for the adsorption procedure, the other steps involved in the method are in general similar to those described in the present investigation. A comparison of the present figures with those of Kodicek' (*loc cit*) shows that the values for maize are of the same order as those

given by him, although the figures for yeast and rice tend to be rather higher. It is interesting to note that Italian millet, which is consumed by the poorer classes in certain parts of India, does not contain any measurable amount of nicotinic acid.

Many further improvements in the method can be made, especially as regards the complete removal of colour in the eluate. The accuracy and the application of the method may further be increased by employing a Pulfrich photometer for the colorimetric estimation. Further work on the application of the method to animal tissues is in progress.

SUMMARY

An adsorption method for the estimation of nicotinic acid content of yeast and other foodstuffs is described. Nicotinic acid is quantitatively adsorbed by medicinal charcoal. By eluting the charcoal adsorbate with hot alcohol-sodium hydroxide solution, an eluate is obtained that contains the vitamin. The charcoal adsorption and elution method is applied to a number of foodstuffs, and added nicotinic acid is quantitatively recovered from the foodstuffs by such treatment.

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NICOTINIC ACID IN BLOOD

BY

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DORFMAN, HORWITZ, KOSER AND SAUNDERS (1939), while experimenting on the effect of the nicotinic acid in blood on the growth of dysentery organisms, observed that growth-promoting activity due to nicotinic acid was confined to the erythrocytes. Pearson (1939) reported that blood plasma from various mammals gave a negative reaction with the cyanogen bromide aniline test. In a previous communication (Kochhar, 1940) the author recorded that only about 5 per cent of the total nicotinic acid content of dogs' blood is present in the plasma. The present investigation was undertaken to discover the distribution of nicotinic acid in the various constituents of blood and whether the estimation of nicotinic acid in blood has any value in the diagnosis of nicotinic acid deficiency.

EXPERIMENTAL

Serum from the clot and plasma from citrated blood were separated as usual. The cells after removal of plasma were further fractionated into two layers by centrifuging at 3,000 r.p.m. for about half an hour. The separation of cells in these two layers was, however, not complete. The red cells in the erythrocyte layers varied from 13.6×10^6 to 18.6×10^6 per μ l. with leucocyte counts of 780 to 6,562 per μ l. The white blood cells in leucocyte layers varied from 49,500 to 446,000 with erythrocyte counts of 2.4×10^6 to 13.1×10^6 per μ l.

The nicotinic acid content of the various constituents of dogs' blood is shown in Table I. The method followed for estimating nicotinic acid or its amide was as described in the previous communication (Kochhar, *loc cit*). It closely resembles the original method described by Swaminathan (1938a, b). The values for the nicotinic acid content of the erythrocyte layers were high. There was a close correlation between the proportion of erythrocytes present in the various test samples and nicotinic acid content, it is probable that nicotinic acid is found only in red

cells The blood plasma contained only a small percentage of the total amount present in the blood

TABLE I

Nicotinic acid μ g per cent in dogs' blood

Sample	1	2	3	4
Whole blood	700	624	704	752
Erythrocyte layer	1,884	1,980	1,606	1,196
Leucocyte layer	1,480	616	540	662
Plasma	<i>Trace</i>	72	115	15
Serum	28	120	62	50

A series of determinations of the nicotinic acid content of the blood of patients suffering from various conditions was undertaken The results are shown in Table II —

TABLE II

Blood nicotinic acid in patients suffering from various diseases

Disease	W b c count per μ l	Nicotinic acid in blood, μ g per cent
1 Lobar pneumonia	7,000	560
2 " "	20,000	400
3 Broncho "	13,000	680
4 " "	11,000	400
5 " "	14,800	416
6 Meningitis	7,900	380
7 "	18,000	380
8 Severe anemia		308
9 " "		348
10 " "		330
11 " "		252
12 Anæmia with diarrhoea		300

The low value for nicotinic acid content in the cases of severe anæmia is of interest. The fact that the nicotinic acid content was not increased in some cases showing a leucocytosis suggests that the leucocytes contain relatively little nicotinic acid.

A pellagrin with marked anæmia was admitted to the Mayo Hospital, Lahore, on 11th March, 1940. He was given 450 μg of nicotinic acid daily for 10 days commencing from 13th March. He showed striking improvement. Subsequently he was given marmite in place of nicotinic acid.

A series of blood nicotinic acid estimations on this patient is shown in Table III. No obvious rise occurred as the result of treatment. It is to be observed that the blood nicotinic acid content in this patient was not much below the average found in normal students and patients not suffering from pellagra, i.e. 367 μg and 355 μg per cent respectively (Kochhar, *loc. cit.*). It therefore appears that determinations of nicotinic acid content of blood are unlikely to be of clinical value.

TABLE III

Blood nicotinic acid in a patient suffering from pellagra

Date	Nicotinic acid in blood, μg per cent
11th March, 1940	384
12th „ 1940	316
13th „ 1940	320
15th „ 1940	380
16th „ 1940	300
18th „ 1940	400
4th April, 1940	412

SUMMARY

1. Most of the nicotinic acid in blood is present in the red cells. Whole blood should be used in the estimation of nicotinic acid content.

2. There was little fluctuation in blood nicotinic acid values in patients suffering from various conditions, including pellagra. Estimation of the nicotinic acid content of blood is probably of little value in the diagnosis of pellagra.

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PATHOLOGICAL CHANGES OCCURRING IN THE PARATHYROIDS IN RATS FED ON A POOR RICE DIET

BY

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In a previous communication from these laboratories (Pal and Singh, 1938), it was reported that when rats were fed on a rice diet resembling that consumed by the poorer classes in India, which has been shown to be deficient in calcium salts (Aykroyd and Krishnan, 1937) changes indicative of hypofunction were observed in the parathyroid gland. When the diet was supplemented by calcium or calcium and phosphorus, the gland remained normal. These experiments, which were mainly devised to study the effect of supplementing rice diets with calcium and phosphorus, lasted for a period of ten weeks only. Further work has been undertaken to investigate changes in the parathyroid gland of rats fed on a similar rice diet for longer periods.

Ragi (*Eleusine coracana*), one of the millets, is widely consumed in India either as a main staple or in conjunction with rice. As this cereal is particularly rich in calcium, the effect on the parathyroids of supplementing a rice diet with ragi was studied.

EXPERIMENTAL

The composition of the basal diet, which is known in the laboratories as the 'cheap Madrassi diet' or the 'poor South Indian diet' (diet I), was as follows —

	Oz	Grammes
Raw milled rice	21 00	596
Dhal arhar (<i>Cajanus indicus</i>)	0 70	20
Black gram (<i>Phaseolus mungo</i>)	0 70	20
Gingelly oil (<i>Sesamum indicum</i>)	0 10	3
Brinjal (<i>Solanum melongena</i>)	1 00	28
Amaranth leaves (<i>Amaranthus gangeticus</i>)	0 50	14
Raw plantain (<i>Musa paradisiaca</i>)	0 50	14
Meat (mutton)	0 06	1 7
Coconut	0 05	1 4

A group of young albino rats (group I), about 50 to 60 grammes in weight, was given the above diet, which was fed *ad lib* in the proportions indicated. The quantities shown are roughly equivalent to the daily intake of an adult man. A second group of animals (group II) was fed on a diet similar in composition, except that four ounces of raw milled rice were replaced by an equal quantity of ragi (diet II). Group III was also given a diet similar to that given to group II, but in this case ragi completely replaced the raw milled rice (diet III). Group IV was fed on the 'stock diet' of the laboratories (diet IV) consisting of 'atta (whole wheat) chapattis' smeared with butter, fresh raw cabbage, fresh raw carrots, sprouted Bengal gram, cow's fresh milk and meat (twice a week). Each experimental group contained 15 animals (8 males and 7 females). The animals were weighed weekly and exposed to sunlight for a few hours daily in the morning time whenever the weather was bright.

The experiment lasted for 55 weeks. The animals were killed after different periods of feeding on the above diets. Immediately after death, the trachea, the œsophagus and the thyroid were removed and fixed together in formol saline. After fixation, the thyroid, with the embedded parathyroids, was dissected out from the other tissues and blocked in paraffin. Serial sections were stained by Ehrlich's acid hæmatoxylin and eosin and, in some instances by Weigert's iron hæmatoxylin and van Gieson's stain.

Certain structural changes have been described in the cells and the connective tissue of the normal parathyroids with advancing age (Cooper, 1925). Accordingly, the histopathology of the glands was studied in comparable age groups, equal numbers of animals from each group being killed simultaneously at different intervals after the commencement of the experiment.

OBSERVATIONS.

The final average weights attained are shown in the Table. Group I grew more slowly and attained a lower final weight than the other groups. Group IV showed the best performance.

TABLE

Average initial and final weights of rats

Group	Initial weight (average) in grammes	Final weight (average) in grammes
I	54.7	150
II	54.7	180
III	54.7	161
IV	57.0	192

The animals, especially those in group I, were examined at frequent intervals for evidences of tetany, e.g. spastic rigidity of the limbs (carpopedal spasm), hyperexcitability, convulsions, etc. None, however, showed these conditions.

The normal parathyroid in the albino rat—The literature on the subject is very scanty and the only available description is that of Hoskins (1924). The following account is mainly based on the author's observations and the data contained in Hoskins' paper—

The parathyroids are separately enclosed within a thin fibrous capsule and are usually attached to the thyroid. In some cases, however, they are embedded in the substance of the thyroid gland. The parenchyma is composed of densely packed epithelial cells. These are polygonal or rounded in shape and are usually arranged in compact masses separated by vascular strands of connective tissue. The predominant type of cell in the parenchyma closely corresponds to the '*transitional variety*' of the principal cells described in the human parathyroid (Gilmour, 1939). This cell contains a round or oval nucleus which is generally centrally placed and stains deeply, the cell walls are not distinct and the cytoplasm at the periphery of the cell is slightly granular. The '*dark*' and the '*water-clear*' varieties of principal cells found in the human parathyroid (Gilmour, *loc cit*) are also seen in the rat parathyroid, but their occurrence and distribution are not always constant. In one specimen, removed from a young animal, the greater part of the parenchyma was made up of the '*water-clear*' type of principal cells. The difference in the three types of principal cells is mainly in the cytoplasm, in the '*dark*' variety, the protoplasm is uniformly deeply stained, while in the '*transitional*' and the '*water-clear*' types only the periphery of the cytoplasm is stained to a varying extent.

It is doubtful whether the oxyphil cells of the human parathyroid have their counterpart in the rat parathyroid. Typical human oxyphil cells are larger than the principal cells and are polygonal in shape, they contain small and often pyknotic nuclei, the cytoplasm stains intensely with acid dyes and contains oxyphil granules. Acidophil cells which do not conform to the above description are, however, seen in the parathyroid gland of the rat. They are often very few in number and their cytoplasm is stained reddish-purple or pink in hæmatoxylin-eosin preparations and contains no noticeable granules.

The amount of connective tissue present in the gland is variable. It is usually scanty and mainly distributed around the vascular channels. In some cases, the amount of connective tissue is so slight that under the low power of the microscope no strands are visible. Closer examination, however, reveals the presence of fibroblasts and delicate fibres of connective tissue around the vessels. Large spindle cells containing yellowish-brown pigment are sometimes present in the perivascular connective tissue, especially round big vessels.

The glands are very vascular. The endothelium lining the capillaries, which appear more like sinusoids is deficient in many places, where the capillaries come into close contact with the parenchymal cells of the gland. A few non-medullated nerve fibres are distributed in the gland along the vascular channels. Colloid vesicles are not seen in the parenchyma of the normal gland.

Changes in the parathyroids of the experimental animals—The animals were killed at the end of 27, 35 and 55 weeks respectively

Group I—(a) The parathyroids of animals killed at the end of 27 weeks were of normal size and showed a preponderance of the transitional type of the principal cells and very few acidophil cells. There was no cytotoxicity in the parenchymal cells and the vascularity of the organs was more or less normal. There was slight fibrosis in the connective tissue framework.

(b) Most of the animals killed at the end of 35 and 55 weeks showed more definite structural changes in the glands. These varied in size, some being smaller than normal and others bigger. The average size was, however, above normal. This was due to hyperplasia of the parenchymal cells. Hypertrophy of the individual cells was not a prominent feature. In most instances, the principal cells appeared normal. Evidences of cytotoxicity were slight. In one animal slight oedema of some of the principal cells was observed and in another, killed after 55 weeks, shrunken cells with pyknotic nuclei were found. Acidophil cells were few in number and normal in appearance.

Striking changes in the connective tissue of the glands were observed. The perivascular connective tissue which is not prominent in the normal gland was increased in amount and, in some cases, dense bands of collagenous connective tissue were found dividing the parenchyma into compact islands of varying sizes. The parenchyma was sometimes further subdivided by fine strands of fibrous tissue connecting the dense bands around the vascular channels. In places the distribution of fibrous tissue was intercellular with the result that the principal cells appeared to be loosely arranged. The amount and distribution of the connective tissue in the glands varied in the different specimens. There appeared to be an increase of pigment-laden spindle cells in the perivascular connective tissue, especially in places where the latter was dense and hyaline.

Serial sections showed that the vascularity of the glands was slightly diminished. In places where perivascular fibrosis was marked the lumen of the vessels appeared very narrow. In some specimens the sinus-like capillaries were diminished in number and less prominent.

As in the normal gland, colloid vesicles were not observed in any instance. No hæmorrhages into the glands were noticed.

Groups II, III and IV—The glands from these animals, killed at the same intervals as those in group I, conformed to the description of the normal gland given in the previous section. There appeared to be a slight increase in the connective tissue of the glands in the older animals, but this remained within normal limits. The glands were very vascular and, in some specimens, the capillaries were rendered more prominent by the congestion of the vessels.

Plates I, II and III illustrate appearances in the normal parathyroid and the various pathological changes described.

DISCUSSION

The most important difference in the experimental diet was in calcium content. The calcium content of the diets I to IV per 2,600 calories (i.e. in terms of human

PLATE I

(All the microphotographs were taken with 'Miflex')

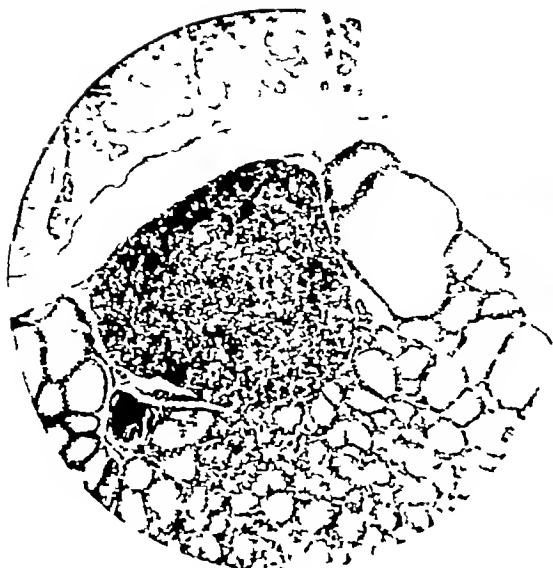


FIG 1

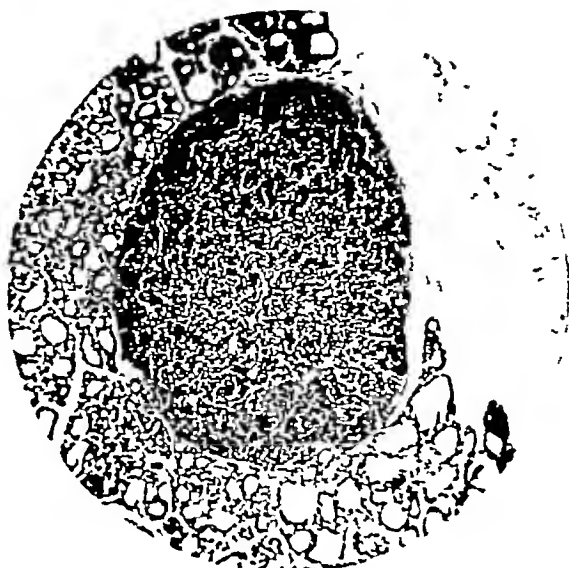


FIG 2

Figs 1 and 2 Low power microphotographs of parathyroid glands of young (Fig 1) and adult (Fig 2) normal rats $\times 60$ Note the preponderance of 'water-clear' type of principal cells in Fig 1



FIG 3



FIG 4

Figs 3 and 4 Low power microphotographs of parathyroids of rats in group I showing the increase in the size of the glands $\times 60$

PLATE II.

(All the microphotographs were taken with 'Mylflex')

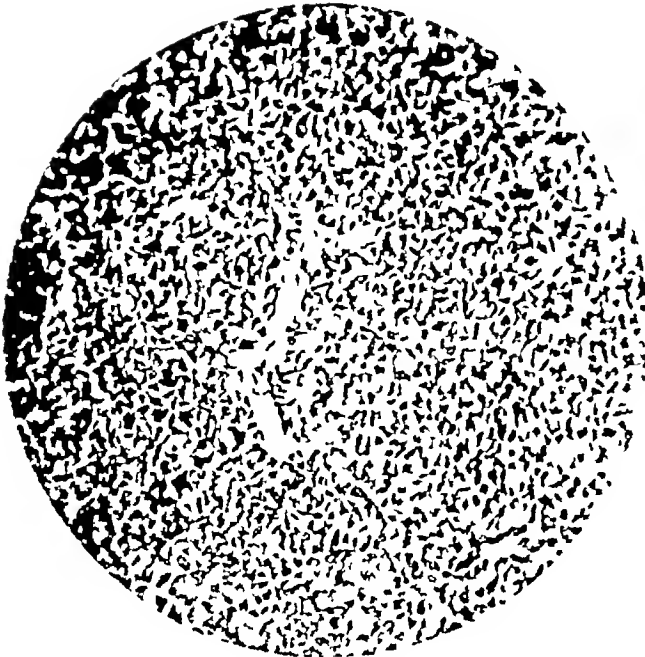


FIG 5



FIG 6



FIG 7



FIG 8

Fig 5 Section of parathyroid of rat in group II showing normal appearances $\times 200$
Figs 6 to 8 Parathyroids of rats in group I showing varving degrees of fibrosis in the glands $\times 200$

PLATE III

(All the microphotographs were taken with 'Miflex')



FIG 9

Fig 9 Section of parathyroid of rat in group I showing irregular fibrosis $\times 200$



FIG 10

Fig 10 High power microphotograph of section of parathyroid of rat in group I showing narrowing of the lumen of the vessels and pigment cells in the perivascular fibrous tissue $\times 400$



FIG 11

Fig 11 Section of parathyroid of rat in group III showing normal appearances $\times 400$



FIG 12

Fig 12 Parathyroid gland of rat in group I showing shrunken cells with pyknotic nuclei $\times 400$

DIETARY AND PHYSIQUE OF MINING POPULATION IN JHARIA COAL FIELDS (BIHAR)

BY

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[Received for publication, June 27, 1940]

INTRODUCTION

THOUGH the actual surface area of the coal mines at present barely exceeds 150 square miles yet the Jharia Mining Settlement includes the whole of the Dhanbad subdivision (787 square miles) of the Manbhum district in Bihar. The settlement resembles an irregular triangle in shape and is situated between the Barakar and Damodar rivers.

The extremely rapid development of the coal industry in Dhanbad subdivision beginning in the closing years of the last century attracted large numbers of people from outside the district. The population increase (221,434 in 1891 Census to 504,736 in 1931) without necessary arrangements for housing, water-supply and sewage disposal led to serious public health problem for the authorities. After various fruitless attempts to improve the sanitation, a statutory body, Jharia Mines Board of Health with its headquarters at Dhanbad, was created in the year 1913. A Medical Officer of Health with subordinate sanitary staff is maintained by the Board. Jharia Water Board, operating from the middle of the year 1924, supplies on the average about 1,600,000 gallons of water daily to a part of the coal field area serving roughly about 123,000 people.

The important vital indices for the last 5 years are given under Table I. The rates seem to be favourable as compared with the respective rates for the province of Bihar.

Analysis of the sickness returns from all the dispensaries in the coal mines for the years 1937-1939 gives the following percentage incidence of the different diseases (assuming the returns for all diseases as 100). Pneumonia, 0.34 per cent, asthma, 0.43 per cent, pulmonary phthisis, 0.05 per cent, all other chest complaints, 9.24 per cent, dysentery, 2.60 per cent, enteric fever, 0.16 per cent,

hookworm and other helminthic infections, 0.19 per cent, diarrhoea, 1.83 per cent, other bowel complaints, 13.06 per cent, influenza, 4.33 per cent, malaria, 23.05 per cent, pyrexia of unknown origin, 5.99 per cent, venereal diseases, 0.19 per cent, and all other diseases, 38.48 per cent. Plague, which was prevalent during the first decade of the present century, has totally disappeared. Cholera, which was responsible for a large number of deaths prior to the installation of water-supply, does occur occasionally in the form of mild epidemics. Smallpox cases occur from time to time.

TABLE I

Certain vital statistics in Jharia Mining Settlement, i.e. Dhanbad Sub-division excluding Dhanbad Municipality

Vital indices	1935	1936	1937	1938	1939
Birth rate	27.08	25.93	27.57	27.21	25.98
Death rate	20.43	16.10	15.50	16.34	15.05
Infant mortality rate	102	108	92	115	99

FAMILIES SURVEYED

An investigation into the food intake of 150 families earning their livelihood from the coal industry was carried out in the autumn and cold weather of 1939, on the lines followed by Aykroyd and Krishnan (1937). Subsequently, another group comprising 44 families, was investigated, this survey being completed during the months of March and April 1940. Hereditary mining families are rarely found as the coal mines in Jharia with few exceptions are barely 50 years old. Even to-day there are miners' families deriving part of their total income from agriculture. No such families have been included in this survey.

Out of a total of 194 families included in this study, 152 belonged to the home province of Bihar, 31 to Bengal, 4 to the United Provinces, 3 to the Central Provinces, 2 to the Punjab, and 1 each to Bombay and Orissa. The total number of families belonging to aboriginal and semi-aboriginal tribes came to 98 and included Bauris, Santals, Bhumiars, Ghatwais, etc.

The families included in this investigation have been classified (see Table II) into four income groups. These are (a) group I, i.e. families with a monthly income up to and not exceeding Rs. 15, (b) group II, i.e. families with monthly income above Rs. 15, and not exceeding Rs. 30, (c) group III, i.e. families with monthly income above Rs. 30, and not exceeding Rs. 50, and (d) group IV, i.e. families with an income above Rs. 50 per month but not exceeding Rs. 200. The average monthly income per family in all the groups works out at Rs. 11.1, Rs. 20.4, Rs. 38.1 and Rs. 82.8 respectively.

For the sake of brevity only the group numbers will be mentioned when referring to any one of the groups later in the text

TABLE II

Classification of families in the different income groups

1	2	3	4	5	6
Group numbers	Number of families in each group	Average monthly income per family	Average number of consumers per family	Average number of 'man value' consumption units per family	Average daily income in annas* per consumer per family
I	74	Up to Rs 15	3 84	2 89	2 30
II	42	Over Rs 15 and up to Rs 30	5 00	3 70	3 28
III	41	Over Rs 30 and up to Rs 50	5 41	4 17	5 58
IV	37	Over Rs 50 but below Rs 200	7 03	5 37	7 71

* One rupee roughly equals 16 annas and consists of 16 annas

ECONOMIC CONDITION

Due to the existence of unemployment most of the miners could not find work continuously on all days of the week and thus their income varied from week to week. The income of each family for 90 days was pooled and divided by 3 to arrive at the monthly income figures. The average consumption units in families belonging to each of the income groups were worked out according to the scale of coefficients prescribed by the League of Nations (1932). It was observed (see Table II) that as income increased the number of dependants in the family also increased.

The occupations followed by principal earner in each family have been classified under four different heads—

(a) Unskilled labour

(c) Supervising staff

(b) Skilled labour

(d) All others

Unskilled labour includes miners (or actual coal cutters), trolley men and loading coolies. Workers in this group were usually paid at the rate of 6 to 8 annas

per ton of coal raised They earned from Rs 6 to Rs 15 per month The skilled labour group consisted of khalasi*, hookman†, coke maker and hammer men (earning about Rs 10 to Rs 25 per month) and loco driver, carpenter, or fitters (Rs 20 to Rs 60 per month) The supervising staff included overman, loading inspector, etc earning a pay of Rs 20 to Rs 60 per month The 'all others' group included doctor, engineer, manager, electrician, etc who earn about Rs 70 to Rs 150 per month and subordinate staff such as clerks, cashiers, compounders, etc who draw a pay of Rs 40 to Rs 100 per month The distribution of the principal earners in each family in the different income groups have been shown in Table III —

TABLE III

Frequency distribution of families in different income and occupation groups

Family groups	Unskilled labour	Skilled labour	Supervising staff	All others	TOTAL
I	69	3	1	1	74
II	24	12	3	3	42
III	8	9	12	12	41
IV	1	1	7	28	37
All groups	102	25	23	44	194

The classification of a few labourers' families in group IV is explained by the fact that in calculating the total income of any family the wages of all earners were pooled together

* Khalasis are technical labourers in connection with pump, boiler, etc

† Hookman works at signals and trolleys

CLASSES OF FOODSTUFF CONSUMED

Intake per consumption unit of the various main classes of food in the different groups is shown in Table IV —

TABLE IV

*Average consumption of various foods in each of the income group
(ounces per consumption unit daily)*

Family groups	Cereals	Pulses	Leafy vegetables	Non leafy veges tablets	Fats and oils	Flesh foods	Milk and milk products*	Fruits and nuts	Sugar and jaggery	Condiments
I	23.6	4.2	2.5	1.9	0.5	1.4	0.4	1.7	0.3	0.6
II	27.4	3.9	1.1	3.6	0.7	1.2	1.6	0.1	0.4	0.4
III	22.8	4.0	0.9	7.0	1.3	1.7	6.2	0.9	0.9	1.0
IV	16.9	3.9	1.4	9.1	2.1	2.3	10.2	2.6	1.1	0.7

* Excludes ghee or butter fat

Cereals—Rice was the chief cereal food consumed and in every family the home-pounded parboiled variety was used. In the case of aboriginal and semi-aboriginal families constituting about 50 per cent of the consumers, the water (marh or conjee) used for cooking was not thrown away. Puffed rice was fairly popular as breakfast or as an afternoon snack. The only other cereal food consumed was wheat flour but its use was limited mainly to the upper income groups. At the time of investigation home-pounded parboiled rice was selling at 18 lb to 22 lb to the rupee and wheat flour at 16 lb.

Pulses—The figures for intake of pulses were found to be higher than those recorded at other industrial areas in Jamshedpur (Mitra, 1940a), or Assam (Wilson and Mitra, 1938). Pulses were selling at 13 lb and 14 lb per rupee respectively.

Leafy vegetables—In the lowest income group the average figures for consumption was the highest, intake in groups II and III was considerably smaller, while that of group IV occupied an intermediate position. In the dietary of industrial workers at Jamshedpur somewhat similar findings have been recorded by the author (Mitra, 1940a), here the quantity of leafy vegetable in the diet was found to decrease with increase of income. There was scarcely any space available at the coohe *dhowrahs* (barracks) for kitchen gardens. Sag or green leaves were selling at half an anna per pound.

Non-leafy vegetables—Potato was a comparatively costly commodity (1 anna per pound). The popular vegetables were bottle gourd (*Lagenaria vulgaris*), brinjal (*Solanum melongena*), broad bean (*Dolichos lablab*), radish (*Raphanus sativas*),

colocasia, ridge gourd (*Luffa acutangula*), nenua (*Luffa ægyptica*), etc. The cost of these per pound was about half that of potato.

Fats and oils—Mustard oil was extensively used. Ghee or butter fat was consumed by the families in the higher income groups. The price of mustard oil was 3-5 annas per pound and that of ghee 10 annas per pound. Butter was not eaten.

Flesh foods—The use of beef was occasional and confined to the 6 Mohammedan and the aboriginal families. Goat's meat (4 annas per pound) was the most popular flesh food. Some poultry meat was included in the diet. In this part of the country fowls sell cheap, two or two and a half annas can purchase a decrepit bird. In a previous communication dealing with the dietary of the aboriginal tribes, the author (Mitra, 1940b) had already shown the aboriginals invariably rear fowls and pigs. So, in such families some amount of pork and chicken was consumed without any increase in the food budget. Fish was selling at 1½ annas to 4 annas per pound and was mostly consumed by the families in the upper income groups.

Milk and milk products—The supply of cow and buffalo milk is limited in extent and sells at ten pounds to the rupee.

Fruits and nuts—The fruits consumed consisted of guava, banana, papaya, etc. The district rarely grows any fruit and fruits have to be imported from other places, consequently they are costly.

Sugar and jaggery—The price of sugar was 3 annas and sugar-cane jaggery 1½ annas per pound. These foods were used mainly to sweeten tea and occasionally as a condiment.

Condiments—These consisted of turmeric, coriander seeds, red chillies, mustard seeds, etc. Onions have also been classified under this head. Condiments were used mostly in the preparation of dhal (gruel from pulses) and curries. They were probably of some value as a source of minerals.

Milk, leafy vegetables, fruits and meat can be considered 'protective' foods. The mean intake of these (Table IV) does not give a satisfactory idea of the trend of consumption in the various groups. Table V shows the frequency distribution of families not including each of these protective foodstuffs in the diet—

TABLE V.

Frequency distribution of families in the different income groups abstaining from the consumption of various 'protective' foodstuffs

Family groups	LEAFY VEGETABLES		MILK AND MILK PRODUCTS		FRUITS AND NUTS		FLESH FOODS	
	Actual	Percentage	Actual	Percentage	Actual	Percentage	Actual	Percentage
I	31	41.9	65	87.8	74	100.0	19	25.7
II	26	61.9	29	69.0	39	92.9	8	19.0
III	25	60.9	13	31.7	24	58.5	10	24.4
IV	14	37.9	3	8.1	3	8.1	7	18.9

The consumption of non-leafy vegetables, milk, fruits, fats and oil, and sugar and jaggery, maintains a positive correlation with the income of the family. This confirms the previous experience of the author (Mitra, 1940a) at Jamshedpur, another industrial centre. Roughly 20 to 25 per cent of the families in each group were vegetarians.

TABLE VI

Frequency distribution of families according to calorie consumption per consumption unit per diem in the different income groups

1	2	3	4	5	6	7	8	9
Family groups	Total number of families	Up to 2,000 calories	Up to 2,500	Up to 3,000	Up to 3,500	Up to 4,000	Over 4,000 calories	Average calorie consumption per man per day
I	74	2	12	18	23	12	7	3,140
II	42	0	4	9	7	7	15	3,571
III	41	1	1	9	13	7	10	3,498
IV	37	0	0	8	18	10	1	3,294

Table VI gives the average intake of calories per man value (consumption unit) per day in all the four groups, as also the frequency distribution, of families in the sliding scale of calorie consumption. The families classed under columns 3 and 4, i.e. with a calorie consumption up to 2,500, were unquestionably obtaining too little food to eat. The lower consumption of calories in the highest income group is accounted for by the fact that these families consisted of people engaged in pursuits requiring only a moderate amount of manual labour. In each of the families in column 3 of the table the average consumption was above 1,900 calories and the maximum average figures under column 8 were in the neighbourhood of 4,500 calories. In one family the energy value of the diet was a little more than 5,000 calories.

Amongst the poorer classes in India, cereals often contribute more than 80 to 90 per cent of the total calorie yield of the diet. Table VII shows the percentage incidence of average intake of calories per consumption unit (man value) *per diem* from cereals, pulses, and fats and oils in different income groups. It is evident that the percentage of the contribution of calories from grain foods gradually diminishes with the increase of income in the family.

The percentage contribution of calories from fats and oils changes in the opposite direction

TABLE VII

Percentage incidence of average intake of calories per consumption unit per diem from cereals, pulses, fats and oils in different income levels

Family groups	Percentage of calories from cereals	Percentage of calories from pulses	Percentage of calories from fats and oils
I	80.32	12.83	3.91
II	77.05	10.42	5.00
III	63.92	10.34	9.69
IV	51.04	11.70	16.15

PROXIMATE PRINCIPLES

The intake of the different nutrient principles in the food per consumption unit per day in each of the families under investigation were calculated from the table of food values contained in Health Bulletin No. 23 (1938) and previous publications by the author (Mitra, 1938, Mitra, Mitta and Roy, 1940) on the subject. Average intake per consumption unit per day in each of the groups is shown in Table VIII —

TABLE VIII.

*Average intake of nutrient principles per consumption unit per day in the different groups**

Family groups	Protein, g	Fat, g	Carbo hydrates, g	Calcium, g	Phos phorus, g	Vitamin A, I U	Vitamin B ₁ , I U	Vitamin C, mg
I	99.2	24.3	612.3	0.716	2.306	4,620	1,035	137
II	104.2	29.7	699.2	0.602	2.630	2,496	1,134	68
III	102.7	53.1	603.2	0.846	2.329	3,724	1,094	101
IV	99.4	70.3	524.1	0.961	2.322	3,730	910	153

* Detailed data may be had on reference to the author

Intake of fat in groups I and II was somewhat low. The average figures for consumption of vitamin A in the families comprising groups II and III was on

the low side. The high vitamin B₁ intake in all the groups is due to the fact that parboiled home-pounded rice, rich in this vitamin, was the chief cereal consumed.

Intake of total protein appeared to be satisfactory. The amount of animal protein consumed was, however, small. Table IX shows the relative consumption of protein and fat of animal and vegetable origin by various groups. It is quite evident that increase in income is followed by a larger inclusion of foods of animal origin.

TABLE IX

Percentage incidence of animal source in case of average figures for protein and fat consumed

Nutrients	Group I	Group II	Group III	Group IV
Protein	7.66	9.58	14.80	21.29
Fat	17.10	19.11	45.60	50.80

The average consumption figures of the important mineral element calcium in groups I, III and IV reached the Sherman standard (0.68 per consumption unit *per diem*), but a closer analysis of the figures reveals a less satisfactory state of affairs. It is evident from Table X that about 55 per cent of the families in group I, 50 per cent in group II, 32 per cent in group III and 8 per cent in group IV were consuming diets whose calcium content failed to reach the standard.

TABLE X

Frequency distribution of families as regards different levels of calcium intake

Family groups	FAMILIES WITH AVERAGE INTAKE OF CALCIUM PER DAY IN MILLIGRAMS			
	Up to 300	Up to 600	Up to 700	Over 700
I	16 (21.6 per cent)	25 (33.8 per cent)	4 (5.4 per cent)	29 (39.2 per cent)
II	4 (9.5 per cent)	17 (40.5 per cent)	8 (19.0 per cent)	13 (31.0 per cent)
III	2 (4.9 per cent)	11 (26.8 per cent)	4 (9.8 per cent)	24 (58.5 per cent)
IV	Nil	3 (8.1 per cent)	4 (10.8 per cent)	30 (81.1 per cent)

The percentage contribution of calories from fats and oils changes in the opposite direction

TABLE VII

Percentage incidence of average intake of calories per consumption unit per diem from cereals, pulses, fats and oils in different income levels

Family groups	Percentage of calories from cereals	Percentage of calories from pulses	Percentage of calories from fats and oils
I	80.32	12.83	3.91
II	77.95	10.42	5.00
III	63.92	10.34	9.69
IV	51.04	11.70	16.15

PROXIMATE PRINCIPLES

The intake of the different nutrient principles in the food per consumption unit per day in each of the families under investigation were calculated from the table of food values contained in Health Bulletin No 23 (1938) and previous publications by the author (Mitra, 1938, Mitra, Mitta and Roy, 1940) on the subject. Average intake per consumption unit per day in each of the groups is shown in Table VIII —

TABLE VIII.

*Average intake of nutrient principles per consumption unit per day in the different groups**

Family groups	Protein, g	Fat, g	Carbohydrates, g	Calcium, g	Phosphorus, g	Vitamin A, I U	Vitamin B ₁ , I U	Vitamin C, mg
I	99.2	24.3	612.3	0.716	2.306	4,620	1,035	137
II	104.2	29.7	699.2	0.602	2.630	2,496	1,134	68
III	102.7	53.1	603.2	0.846	2.329	3,724	1,094	101
IV	99.4	79.3	524.1	0.961	2.322	3,739	910	153

* Detailed data may be had on reference to the author

Intake of fat in groups I and II was somewhat low. The average figures for consumption of vitamin A in the families comprising groups II and III was on

TABLE XII

Height and sitting height in inches and weight in pounds of children in coal fields

Age	Boys				Girls			
	Number examined	Height	Weight	Sitting height	Number examined	Height	Weight	Sitting height
Up to 3 years	44	32.5	20.1	17.7	38	31.8	19.6	17.6
" 4 "	52	36.4	24.7	19.0	42	36.0	24.1	19.0
" 5 "	72	38.7	28.4	20.4	51	37.7	25.2	20.2
" 6 "	122	41.8	32.5	21.9	65	41.1	30.7	21.5
" 7 "	126	44.7	36.7	22.6	86	43.1	34.6	22.4
" 8 "	137	46.4	40.3	23.4	66	45.5	38.2	23.4
" 9 "	152	47.7	43.6	23.8	51	46.8	40.5	24.0
" 10 "	122	49.5	47.1	25.0	32	49.3	43.9	24.5
" 11 "	69	51.8	52.5	25.7	24	50.7	49.0	25.4
" 12 "	77	53.0	54.9	26.0	13	52.5	53.3	26.0
" 13 "	69	55.7	65.2	27.2	11	52.9	57.8	27.0

The means are similar to those recorded for Madrassi and Assamese children (Aykroyd and Krishnan, *loc cit*, Wilson and Mitra, *loc cit*) but below those of children in Najafgarh, Delhi province (Shourie, 1939). The boys were found to be taller and heavier than the girls in the same age group.

CLINICAL EXAMINATION

The state of nutrition of the children was assessed clinically according to the standard described previously by the author (Mitra, 1940a) as

good', 'fair' and 'poor' The results of the examination are shown in Table XIII —

TABLE XIII

Rating of state of nutrition of children by clinical examination

Sex	Number examined	Good, per cent	Fair, per cent	Poor, per cent
Boys	1,042	2 40	73 90	23 70
Girls	470	2 30	78 08	19 62
All children	1,521	2 37	75 21	22 42

All these children were further examined for the presence of xerophthalmia, phrynoderma and angular stomatitis. These conditions are believed by a large majority of workers to be associated with intake of food deficient in quality. As any one child may suffer from more than one deficiency condition column (e) of Table XIV gives the percentage of children manifesting either one or more than one signs of deficiency. The girls were found to be in a better state of nutrition than the boys. The children were also examined for the incidence of caries and malocclusion of teeth. It was found that 10 36 per cent of the boys and 7 52 per cent of the girls showed evidence of gross caries visible on rapid inspection without the use of probe or mirror, while 37 71 per cent of the boys and 38 20 per cent of the girls displayed some degree of malocclusion.

TABLE XIV

Percentage incidence of clinical signs supposed to be associated with deficient dietary

(a)	(b)	(c)	(d)	(e)
Sexes	Phrynoderma, per cent	Xerophthalmia, per cent	Angular stomatitis, per cent	Percentage of children suffering from (b), (c) or (d), per cent
Boys	6 05	7 96	6 05	20 1
Girls	5 01	4 17	6 05	15 0
All children	5 72	6 77	6 05	18 5

KNUDSEN-SCHIOTZ SIGN

Bigwood (1937) in his note on the 'Method of assessing the state of nutrition of children and adolescents considered in relation to defective diet' mentions the examination of the dorsal median furrow as a test for the state of nutrition. It has been claimed that in the subjects suffering from defective nutrition the median furrow (locating the tips of the spinous processes of the vertebræ and the inter-connecting ligaments) is broken or unduly sinuous. In the present study all the children (1,042 boys and 479 girls) previously referred to were particularly examined for this sign and those with broken or unduly sinuous median furrow have been classed as 'unsatisfactory' and the rest as 'satisfactory'. According to this criterion 285 boys and 136 girls have been rated as 'unsatisfactory' or malnourished (Table XV).

By this method a higher percentage of children was selected as 'malnourished' as compared to the clinical rating by naked-eye examination or incidence of signs of deficiency. Further work on this test is necessary before any conclusions as to its practical value can be drawn.

TABLE XV

Knudsen-Schiotz index of the children
(*Examination of dorsal median furrow*)

Sex of children	EXAMINATION OF DORSAL MEDIAN FURROW		
	Satisfactory	Unsatisfactory	Total number of children
Boys	757 (72.6 per cent)	285 (27.4 per cent)	1,042 (100.00 per cent)
Girls	343 (71.6 per cent)	136 (28.4 per cent)	479 (100.00 per cent)
All children	1,100 (72.3 per cent)	421 (27.7 per cent)	1,521 (100.00 per cent)

SUMMARY

1. A diet survey of 194 families employed in a coal mining settlement in Bihar was carried out in September to December 1939, and March to April 1940. The families were divided into four income groups with monthly income per family of (a) up to Rs 15 (b) over Rs 15 to Rs 30, (c) over Rs 30 to Rs 50 and (d) above Rs 50 but not exceeding Rs 200. With increase in income the number of dependants in the family also increased.

2 Average calorie intake per consumption unit was satisfactory but 20 and 10 per cent of families in the lowest income groups respectively had an intake of less than 2,500

3 Consumption of milk, fruits and meat increased with increasing income. On the other hand, the poorest families consumed more leafy vegetables. The diets of a considerable percentage of families were deficient in vitamins A and C.

4 The state of nutrition of 1,521 children was investigated by clinical assessment and by recording the presence or absence of xerophthalmia, angular stomatitis and phrynoderma. Some 22 per cent were found to be in a poor state of nutrition by method of clinical assessment, while about 18 per cent were suffering from deficiency diseases (xerophthalmia, phrynoderma and stomatitis).

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INVESTIGATIONS INTO THE EPIDEMIOLOGY OF EPIDEMIC DROPSY

Part X

A NOTE ON AN OUTBREAK OF EPIDEMIC DROPSY ASSOCIATED WITH THE USE OF MUSTARD OIL PRESSED FROM SEEDS ADULTERATED WITH SEEDS OF *ARGEMONE MEXICANA*

BY

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[Received for publication, July 11, 1940]

THE scene of the outbreak was a remote village in Northern Bengal. It is entirely an agricultural community consisting of 89 persons belonging to 11 families. The people have few wants and, therefore, have little contact with the outside world. Only one person amongst them was regularly in contact with another village where he served as a teacher. He lived there during the week days and came home on week-ends and holidays. The monotony of isolated life was broken only twice a week when villagers visited the *hât* (bi-weekly market) held in a neighbouring village about two miles away.

So far as could be ascertained, general health of the people, prior to the outbreak, was good, at any rate, and no cases of epidemic dropsy had occurred in the village or in the neighbourhood. The present outbreak commenced on the 7th April, 1940, when two families were affected. Three other families were affected later. A sketch plan of the village showing the location of houses occupied by different families is shown in the Map. Details regarding the number of persons in the families and the dates of the occurrence of the first cases are shown in Table I. Since the investigation was carried out about 2 months after the beginning of the

SKETCH MAP

OF THE VILLAGE SHOWING AFFECTED AND
UNAFFECTED FAMILIES AND THE DATE
OF ATTACK OF THE FIRST CASE

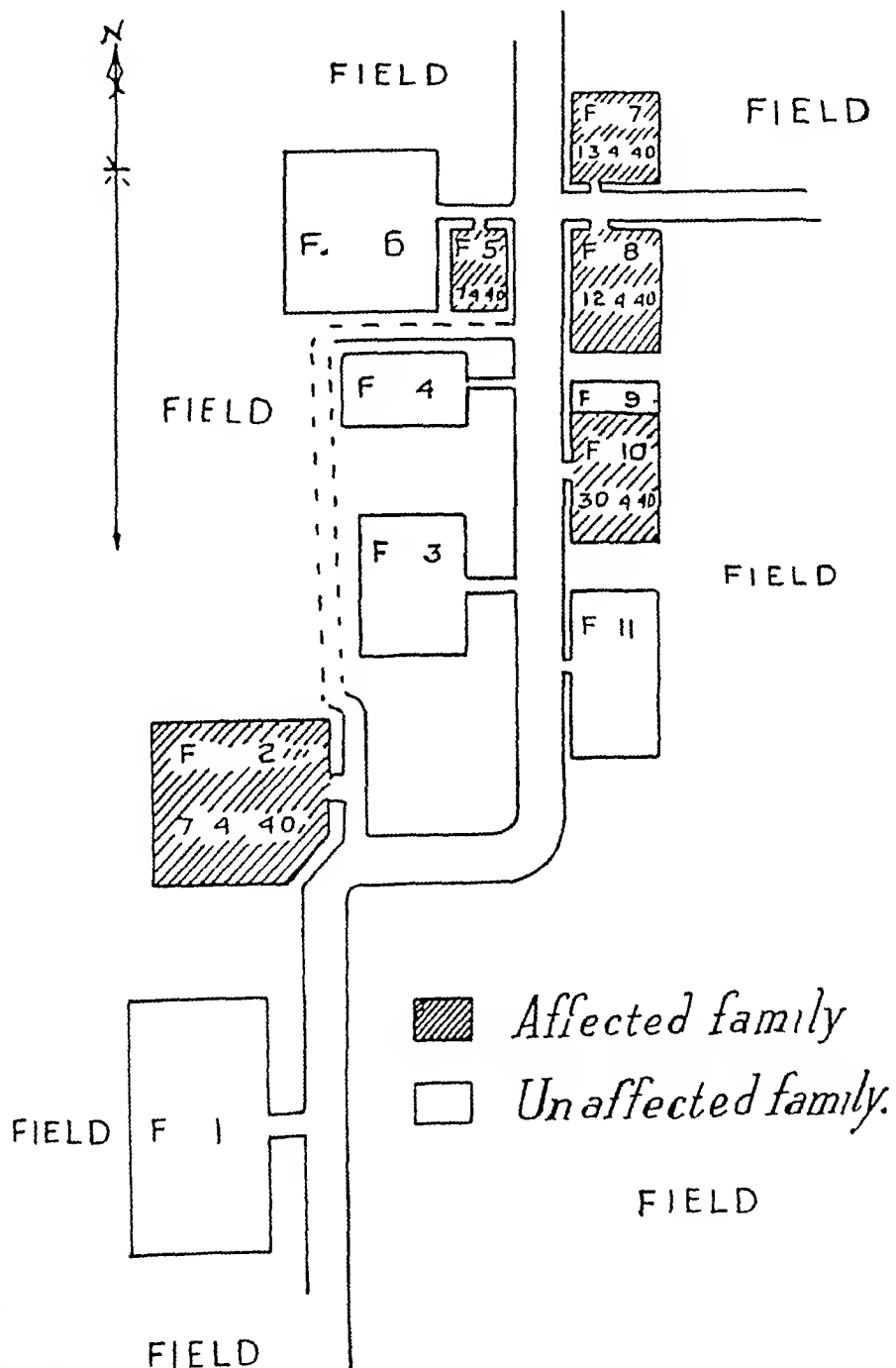


PLATE IV



Fig 1 12 x 6 Skin section (b) shows flattening of papillae, loosening of fibres due to oedema and bunches of young and dilated capillaries with perivascular infiltration



Fig 2 12 x 40 Skin section (a) shows dilated interpapillary vessels, increase of collagen and perivascular infiltration



Fig 3 12 x 40 Skin section (b) shows flattening of papillae, increased collagen and bunches of young and dilated capillaries



Fig 4 12 x 40 Skin section (a) shows large amount of collagen matter and extensive extravasation of blood

outbreak, it was not found possible to work out in detail the chronological order of all the cases

TABLE I

Chronological order of occurrence of first cases in the families

Family number	Date of onset of first case in the family	Number of family members	Number of visitors	Number of cases in the family
2	7th April, 1940	4	1	5
5	7th " 1940	5		5
8	12th " 1940	3		3
7	13th " 1940	5		5
10	30th " 1940	7		6
TOTALS		24	1	24

As will be seen from Table I, a remarkable feature of the outbreak is that every member of the affected families save one suffered from the disease, while none of the members of the other families was attacked although these families were occupying adjacent houses. The single exception was a young married woman who had been away from the village for a considerable time prior to the outbreak and who did not rejoin the family till a few days after the occurrence of the last case in the family. The visitor was residing in the affected family during the epidemic and became ill soon after returning home in another village. She developed mild symptoms. In a small village community like this there is naturally a considerable amount of personal contact, particularly when all the villagers belong to one community as in the present case.

At the time of our visit, out of 24 cases 17 were still ill exhibiting typical signs and symptoms of epidemic dropsy. An analysis of results of clinical examination are given in the *Appendix*.

Pieces of skin sections were obtained in two biopsies. The microscopic appearance of skin sections (as seen in Plate IV, figs 1, 2, 3 and 4) is typical of epidemic dropsy (*vide* Shanks and De, 1931, Chopra, Chaudhuri and Panja, 1935, Lal, 1938). There is a thick deposit of melanin in the deeper layer of the *rete*. Papillae are flattened out in some parts due to interpapillary oedema. Elsewhere they are well marked. Interpapillary vessels are dilated and contain blood corpuscles. There are many young capillaries with their lining endothelial cells rich in protoplasm. Some dilated vessels are seen in the *rete mucosa*. Collagen is thick and fibres are loose and oedematous. Bunches of dilated young capillaries are seen all through the subcutaneous tissue and in the adipose layer. There is a considerable amount of perivascular infiltration. In some parts of the subcutaneous tissue there is a large amount of extravasated blood.

Age and sex distributions of cases and non-cases are shown in Tables II and III —

TABLE II

Age distribution of cases and non-cases

Age groups —	0—	1—	1—	3—	5—	15—	25—	35—	45—	55—	65—	Total
Cases	0	0	0	0	0	5	1	7	1	1	0	24
Non cases	0	0	4	4	21	18	8	5	2	1	2	65

TABLE III

Sex distribution of cases and non-cases

Sex —	Male	Female	Total
Cases	12	12	24
Non cases	28	37	65

There were no infants in the families

The sources from which the chief articles of food, namely rice and oil, were obtained are shown in Table IV —

TABLE IV

Sources of supply of rice and mustard oil of the affected and unaffected families.

Families	SOURCES OF RICE SUPPLY				SOURCES OF MUSTARD OIL SUPPLY	
	From own paddy	Paddy purchased from other villages	Purchased rice from other villages	Begging	Local <i>Andu</i>	By weekly market
Affected	3	1	1	0	5	0
Unaffected	5	0	0	1	0	6

The dates on which the different families purchased oil prior to and during the outbreak could not be definitely ascertained, nor the exact information regarding the amount of oil purchased each time could be obtained. However, the fact remains that whatever oil the affected families consumed was obtained from the

local *lôlu* (oil-presser) and that the entire product of the *lôlu's ghami* (oil mill) was consumed by these families which included his own. It was only after a large number of cases had occurred amongst this clientele and the oil had come under suspicion that the affected families sought other sources of supply.

DISCUSSION

The members of the affected and the unaffected families mixed with each other very freely before, during and after the outbreak. Local inquiries showed that there were no social or economic barriers amongst them. The sources of rice for each family were different, no two families having a common source. On the other hand, there was a sharp distinction between the affected and unaffected families as regards the sources of mustard oil. While all the affected families obtained their mustard oil supply from the village *lôlu*, the six unaffected families obtained their supply from different dealers in the bi-weekly markets. The reason for this difference was not clear in the beginning but on careful inquiry the fact was brought out that up to September last year all the families patronized the village *lôlu* but in that month an altercation occurred between the village headman and the *lôlu* because the latter had insulted the former in the market by demanding his dues on account of the oil supplied. The headman boycotted the *lôlu* and was able to persuade five other families to purchase their oil elsewhere. The other five families either on account of the influence of another headman who was particularly friendly to the *lôlu* or because of convenience continued to purchase the oil from him. Sometimes during the last month of March the *lôlu* had obtained a supply of about 12 pounds of seeds of *Argemone mexicana* from some Santal boys. On *lôlu's* own admission these seeds had somehow got mixed up with his stock of mustard seed. The resulting mixture contained a high percentage of *Argemone* seeds. Since, however, a sample collected on 12th May, 1940, still contained 28 per cent of *Argemone* seeds, it would appear that the practice of adulteration had continued for some time. The *lôlu* with whom oil-pressing was only a supplementary industry used to press about 20 pounds of seeds at a time and not more than 40 pounds in a day. His total produce amounted to 5 pounds to 10 pounds of oil a day. He, however, worked the *ghami* according to requirements and sold the oil only locally. A small portion of the oil, a remnant of the stock pressed early in April, contained 5.7 per cent of *Argemone* oil as determined by the colorimetric method (*vide* Lal, Mukherji, Das Gupta and Chatterji, 1940). Since, however, it had been kept in a glass phial in diffused light, the chances are that the fresh oil contained a much higher proportion of the foreign oil. A sample of oil seized from an affected family contained only 3.1 per cent of *Argemone* oil. However, on investigation it was found that this sample of oil was not a part of the oil obtained from the *lôlu* but it was a sample of oil purchased elsewhere in the same bottle in which the *lôlu's* oil had been stored.

The important facts connected with the outbreak may be summarized thus —

- (1) There was a close contact amongst all the villagers all through
- (2) The source of rice supply was individual

- (3) Mustard oil consumed by all the affected families was obtained from a common source. The unaffected families purchased their mustard oil elsewhere.
- (4) Within a fortnight previous to the outbreak mustard seed from which the oil was expressed for distribution amongst the would-be affected families was grossly adulterated with seeds of *Argemone mexicana*.
- (5) The oil pressed from the adulterated seeds presumably contained over 5.7 per cent of *Argemone* oil.
- (6) The highly contaminated oil was used by the affected families for a period of at least 10 days before the outbreak in quantities estimated at $\frac{3}{4}$ ounce or less per head per day. At the lowest estimate each affected individual prior to sickness consumed *Argemone* oil representing 25 mg of the reacting substance. The actual amount might have been twice this figure or even more.
- (7) That in all probabilities the practice of adulteration continued for some time subsequent to the occurrence of cases.
- (8) All the persons who partook of the contaminated oil suffered from a disease exhibiting typical signs and symptoms of epidemic dropsy. None of the persons who did not consume the oil suffered.
- (9) The disease was of a severe type both in its clinical manifestations and as regards the number of persons involved.
- (10) Besides contamination of the mustard oil no other factor common to the affected families which might have been introduced recently could be discovered.

In view of the observations previously reported by us (Lal, *loc cit*, Lal and Roy, 1937, 1939, Lal, Mukherji, Roy and Sankaran, 1939, Lal *et al*, 1940) and by other workers (Chopra, Pasricha, Goyal, Lal and Sen, 1939) the inference that the disease was caused by the ingestion of mustard oil contaminated with oil of *Argemone mexicana* is obvious. This outbreak furnishes perhaps as close a parallel of a deliberate, well-controlled experiment conducted to incriminate *Argemone* oil as could possibly be expected.

A comparison of the results of a human experiment (Lal and Roy, 1939) and the observations made in the present outbreak draws attention to an important fact. While the average period of the appearance of first cases after the commencement of the adulterated oil was probably about the same in the two instances, the severity of the disease in the village cases was much greater than in the experimental subjects even though the former had consumed the oil in much smaller quantities. One of the reasons of this difference may partly be that the villagers being ignorant of the cause of their trouble continued the use of the toxic oil for a long time but there is also an obvious explanation, namely the difference in the concentration of *Argemone* oil in mustard oil. The oil used in the experiment contained 4.0 per cent of the toxic oil, while the *kôlu's* oil contained 5.7 and probably higher percentage of *Argemone* oil. As a rough estimate, the average total amount of poison represented

by the reacting substance consumed by the victims of the outbreak probably amounted to twice or more of that taken by the experimental subjects

SUMMARY

1 An outbreak of epidemic dropsy which occurred in a remote part of Bengal where the disease had not been known previously has been described

2 It was confined to a group of five families all but one member of which including a visitor were affected. The single person who formed the exception had been away and did not return till a few days after the occurrence of the last case in the family. The disease was of a severe type involving 24 persons. Three cases ended fatally.

3 A factor common to all the affected families and not so to the unaffected families was the source of mustard oil. The opportunity of contact between the members of the affected and unaffected families was apparently the same as between the members of the families of the two groups. Source of rice was individual for each family, whether affected or not.

4 Prior to the outbreak there was gross adulteration of mustard seeds with *Argemone* seeds in the house of the *lôlu* who regularly supplied mustard oil to the families subsequently affected including his own.

CONCLUSION

The facts revealed in the investigation strongly support the conclusion that the consumption of mustard oil containing *Argemone* oil gave rise to an outbreak of epidemic dropsy of a severe type. The incriminated oil which contained at least 5.7 per cent of *Argemone* oil was consumed, on an average in amounts ranging from $\frac{1}{2}$ oz to $\frac{3}{4}$ oz daily for 10 days or more by most of the victims.

ACKNOWLEDGMENTS

We have much pleasure in expressing our thanks to Mr H P Nath, Sanitary Inspector, for drawing our attention to this outbreak and for the invaluable assistance rendered by him in conducting the investigation. We are also grateful to Dr M M Barory for his assistance in obtaining clinical material from the patients.

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APPEN

CLINICAL EXAMINA

*All the 26 persons in the affected families gave history of intestinal disturb
Of these 12 were examined in detail The main signs and symptoms*

Family number	Age in years	Sex	Date of attack	Date of examination	Initial symptoms	CHIEF		
						Diarrhoea	Fever	Oedema
2	55	Male	12th April, 1940	6th June, 1940	Diarrhoea	+	+	+
5*	35	"	7th " 1940	6th " 1940	"	+	+	+
5	42	Female	20th " 1940	7th " 1940	"			+
5	18	"	20th " 1940	7th " 1940	"			+
7	18	Male	13th " 1940	6th " 1940	"	+		+
7	8	Female	13th " 1940	6th " 1940	"	+	+	+
7†	18	Male	16th " 1940	6th " 1940	"			+
7	40	Female	14th " 1940	7th " 1940	"		+	+
8	39	Male	20th " 1940	6th " 1940	"	+	+	+
8	8	Female	15th " 1940	6th " 1940	"	+	+	+
8	19	"	22nd " 1940	7th " 1940	"		+	+
10	8	Male	2nd May, 1940	7th " 1940	"			+

* Skin section (a)

† Skin section (b)

DIX

TION OF CASES

ances and fever followed by œdema of legs, pigmentation and breathlessness exhibited by the cases whose histories were recorded are shown below --

PRESENT SIGNS

Hyperpigmentation	Rash	Palpitation	Dyspnoea	Cough	Enlargement of heart	Soft bruit replacing first sound in mitral area	First sound muffled	Accentuated pulmonary second sound	Bleeding from rectum
+		+	+		+		+	+	+
+		+	On exertion	+	+	+		+	
+		+			Refused examination				
+	+	+					,		
+	+	+	On exertion		+	+		+	
+		+			+			+	
+		+	On exertion		+	+		+	
+		+	On exertion		Refused examination				
+		+	On exertion		+		+	+	+
+		+	On exertion		+	+		+	
+		+	On exertion	+	Refused examination				
+		+	On exertion						

INVESTIGATIONS INTO THE EPIDEMIOLOGY OF EPIDEMIC DROPSY

Part XI.

BIOLOGICAL TEST OF SPECIFIC TOXIN IN SAMPLES OF OIL

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THE development of the physical and chemical tests, reported in a previous communication (Lal, Mukherji, Roy and Sankaran, 1939), by means of which supplies of mustard oil containing the toxic substance responsible for the epidemic dropsy syndrome can be detected marks an important advance in our knowledge on which practical control of the disease should be based but the chemical nature of the toxic substance still remains undetermined. Apart from the theoretical interest, this unsolved problem came unexpectedly to the fore in connection with our endeavours to develop a test for quantitative estimation of the toxicity of oil. As reported in Part IX of this series the reacting substance in the toxic oil suffers a progressive diminution when the latter is exposed to sunlight, especially

in the presence of air. The question naturally arises whether or not the loss in the reacting element of the oil is accompanied by a corresponding reduction in its toxic contents. The answer to this question will naturally depend upon the relationship which exists between the reacting molecule and the toxic factor. If the two are identical, the quantitative chemical test will be a faithful indicator of the toxicity of the oil. The same will be the case if two different substances are responsible for toxicity and reactivity but both are present in the oil *always* in the same proportion and are equally susceptible to the influence of light and air and of other possible factors adversely affecting them. If, on the other hand, neither of these propositions holds good, the value of the chemical test will be limited and the quantitative test will lose all its significance. To clear this point and indeed to solve a number of issues in connection with the nature of the toxic substance and its relation to the reacting substance appeal has finally to be made to the clinical test on human subjects. However, as previously mentioned, facilities and opportunities for conducting human experiments are extremely restricted. Under the circumstances the search for other biological tests becomes imperative. Reference has already been made (Lal *et al*, *loc cit*) to the unsuccessful attempts made for the development of a skin test on human subjects. The problem, therefore, resolved itself to a search for an animal which might react to the toxic substance responsible for the symptoms of epidemic dropsy in a manner identical or similar to human subjects.

Two series of feeding experiments on animals have been described in Part VI of the series. The animals used were rats, cats and monkeys. The results indicated that allylthiocyanate had no effect on these animals unless added in excessive quantities when it caused some loss in weight. On the other hand, a supply of mustard oil (to be called 'S' for short), which had been incriminated on epidemiological grounds, proved 'toxic' to rats in the first series of experiments inasmuch as it caused reduction in weight and early death. Heat did not reduce the toxic properties of the oil. However, it failed to produce signs and symptoms of epidemic dropsy as seen in man. Another suspected supply of mustard oil ('K' oil) was found to be 'non-toxic'. An interesting fact which emerged from the second series of experiments was the 'non-toxicity' of 'S' oil which was then about a month older than at the time of first experiment. It may be mentioned here that definite experimental evidence of toxicity to human subjects was lacking both in the case of 'S' and 'K' oils. Monkeys refused food containing oil and were, therefore, unsuitable for feeding experiments. Cats were the best animals in this respect. Since, however, no symptoms resembling epidemic dropsy could be induced in the animals used in experiments so far reported much confidence could not be placed on the biological test for estimating toxicity of oils or their fractions in respect of epidemic dropsy. Now that we have good reasons to believe that the source of the toxic substance is argemone oil, the problem has assumed a different aspect. While formerly we were not in possession of sufficient quantities of oil of proved toxicity which we could employ in animal experiments, we could now prepare mixtures of different grades of toxicity as desired. The problem has, therefore, been further investigated.

In the absence of our ability to induce signs and symptoms of epidemic dropsy in experimental animals, three criteria for suggesting the toxicity of oils or their fractions are available —

- 1 Rate of change in weight in growing animals
- 2 Time to death
- 3 Histological changes in tissues

EXPERIMENTS ON RATS

The following materials were used in these tests —

- 1 Pure mustard oil obtained from a local jail
- 2 Since mustard oil itself was not wholly agreeable to rats some rats were fed on ghee (clarified butter) to see if by this means proper growth of the animals could be obtained
- 3 Pure argemone oil
- 4 Ten per cent argemone oil in mustard oil
- 5 A sample of epidemiologically incriminated mustard oil which had been proved toxic by means of a human experiment (Rangpur oil, *vide* Lal and Roy, 1939)
- 6 A sample of suspected mustard oil of unknown toxicity obtained from the field ('suspected oil')

The basic diet on which the rats were fed was the same as used in the previous experiments (*vide* Lal, Ahmad and Roy, 1938)

(1) *Time to death* *—In many of the groups not excepting those put on ghee and mustard oil some of the rats died early. From the post-mortem appearance these fatalities appeared to be non-specific. Moreover, in the experiment with Rangpur oil (group V) the rats were killed at regular intervals to watch the progress in pathological changes if any. For these reasons it is not possible to obtain any indication of toxicity or otherwise of any of the materials tested, on the basis of time to death. All that can be said is that healthy animals can live for long periods when fed on the materials tested.

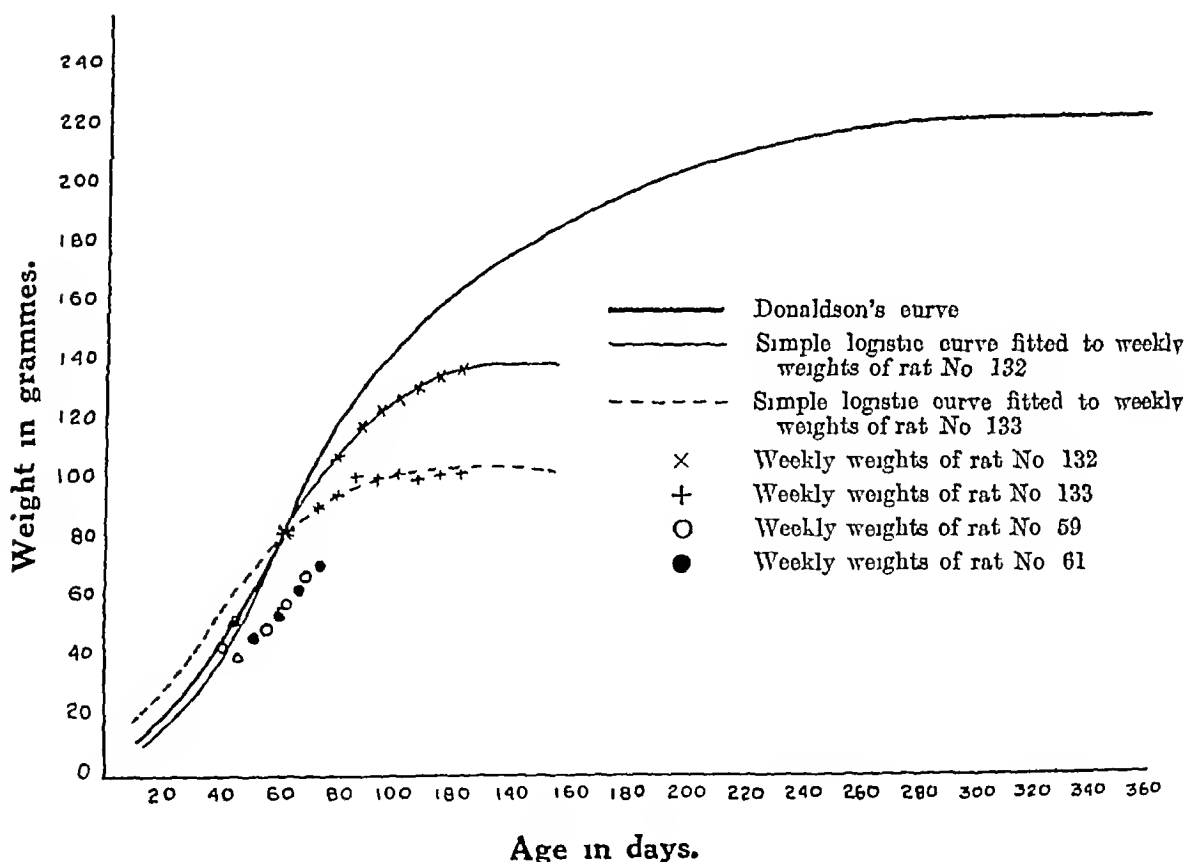
(2) *Rate of change in weight* —It is usual to give the average changes in weight during the experimental period for each rodent. While these figures in a rough way give indications of the toxicity of the materials tested, they fail to convey the whole information because in the first place the rate at which the rats gain weight will depend upon the age or the growth phase of the animal which fact is not taken into account and in the second place we do not get any indication as to the stage

* Detailed data have been omitted to save space. Those desiring full details may write to the author.

of the experiment at which loss in weight, if any, takes place. Unfortunately, we did not possess correct information about the exact age of the rats used nor did we have any experimental data regarding the growth curve of the normal rats under the dietetic and environmental conditions to which they were subjected during the experiments. However, the rats used were *Mus norvegicus* and belonged

GRAPH 1

Growth of female rats fed on pure mustard oil



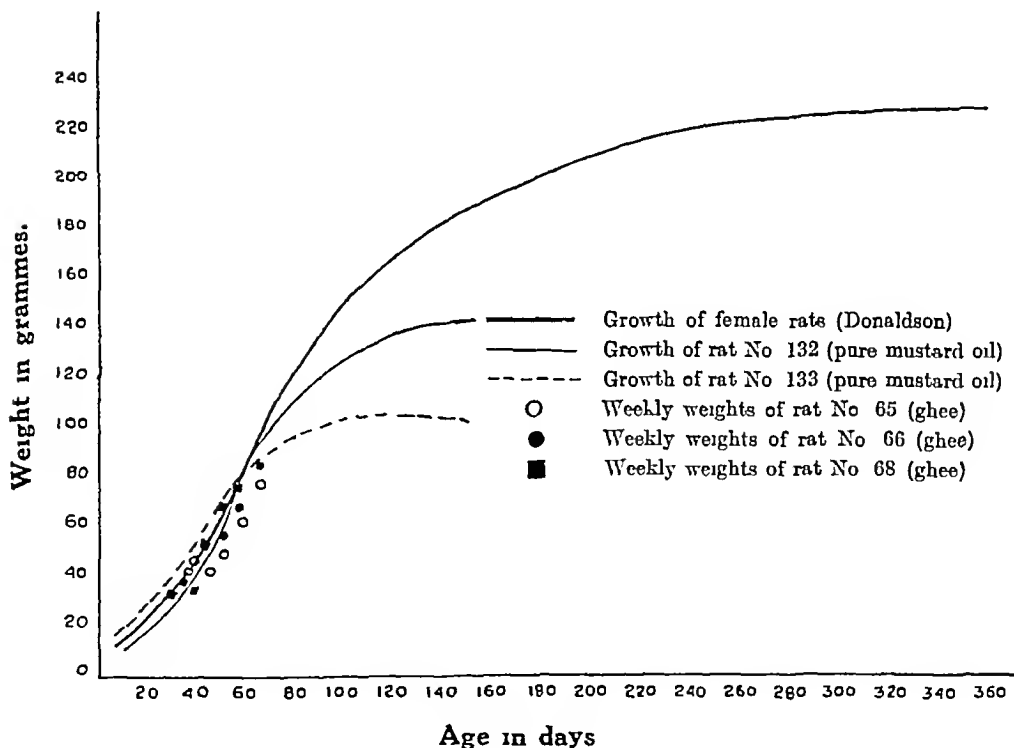
to albinus strain and, therefore, we have assumed that prior to the experiments the female animals were subject to the growth curves described by Donaldson (1924). In the case of male rats, the skew logistic curve devised by Pearl (1926) from Donaldson's data was assumed.

The problem therefore may be stated as follows —

- 1 Whether rats treated with pure mustard oil follow logistic curve of growth and if so whether the curve is identical or significantly different from the expected curve of growth subsequent to their commencement of mustard oil diet

GRAPH 2

Growth of female rats fed on ghee



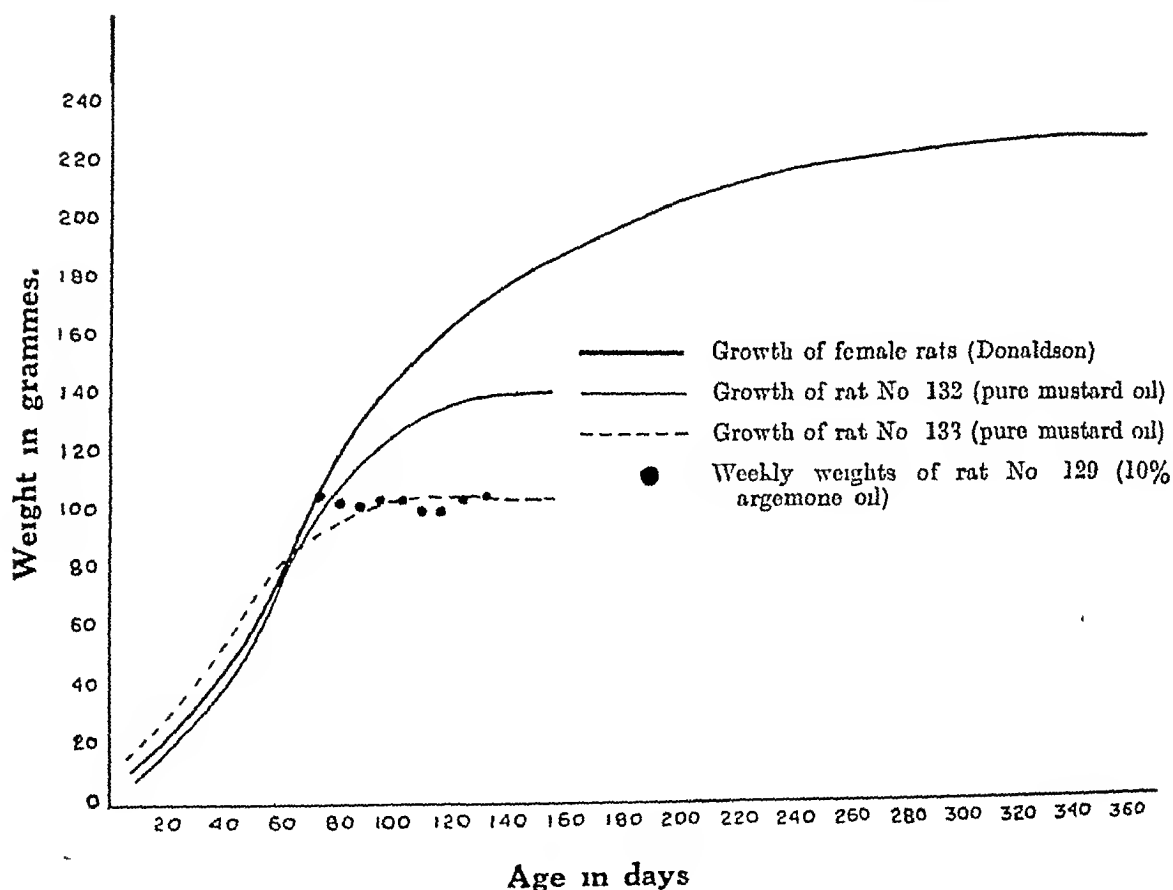
- 2 Whether rats put on other materials follow logistic curve of growth and if so whether their curves of growth significantly differ from the curve of growth of 'pure mustard oil rats'

In Graph 1 is shown the Donaldson curve of growth for female rats. In general appearance Donaldson's curve resembles closely the simple logistic curve

like the logistic curve fitted to weekly weights against assumed age of two female rats put on mustard oil (Nos 132 and 133) It also resembles the curve joining the points denoting the weekly weights against assumed ages of the other two rats put on mustard oil (Nos 59 and 61)

GRAPH 3

Growth of a female rat fed on 10 per cent argemone oil in mustard oil

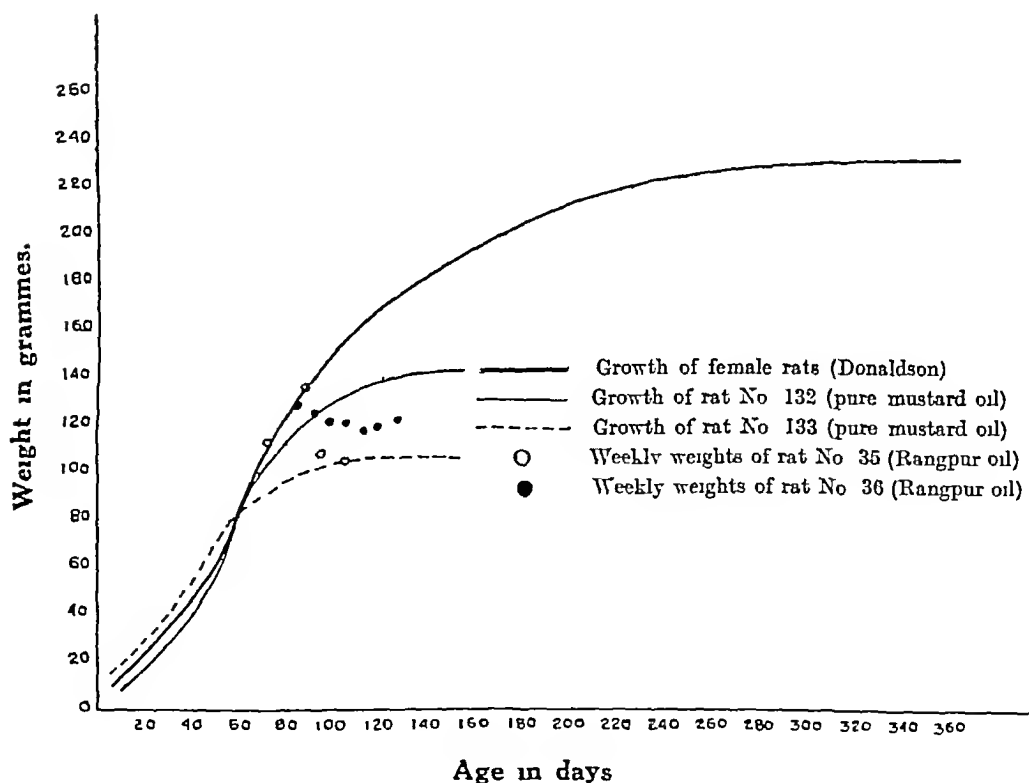


In Graphs 2, 3, 4 and 5 are shown the weekly weights against assumed ages for female rats put on ghee, on 10 per cent argemone oil, on Rangpur oil and on 'suspected oil' respectively. Corresponding graphs for male rats are Nos 6 (pure mustard oil), 7 (ghee), 9 (10 per cent argemone oil), 10 (Rangpur oil) and 11

('suspected oil') For purposes of comparison, on the graphs of male rats is depicted the skew growth curve of Pearl fitted on Donaldson's data for male rats together with two dotted curves showing the range of variation equivalent to twice the standard deviation

GRAPH 4

Growth of female rats fed on Rangpur mustard oil



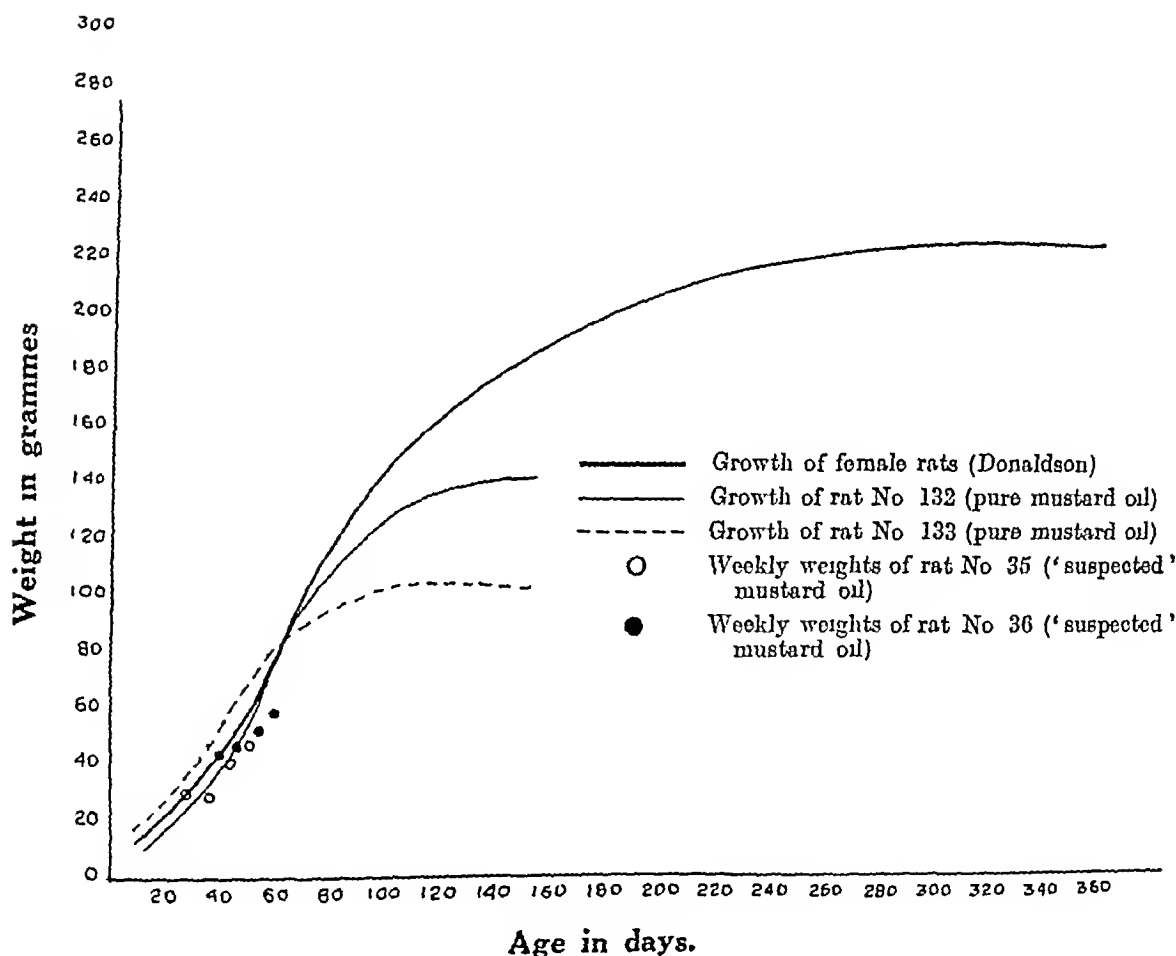
The *Appendix* contains a discussion of the mathematical reasoning regarding the nature and significance of the various observed curves of growth, to which reference may be made for a critical examination of the statements which follow

However, the main facts are sufficiently obvious to be appreciated by a perusal of the graphs, namely —

In the case of female rats (Nos 132 and 133) the growth curves are consistent with the logistic law, but the two curves are materially different from one another

GRAPH 5

Growth of female rats fed on 'suspected' mustard oil

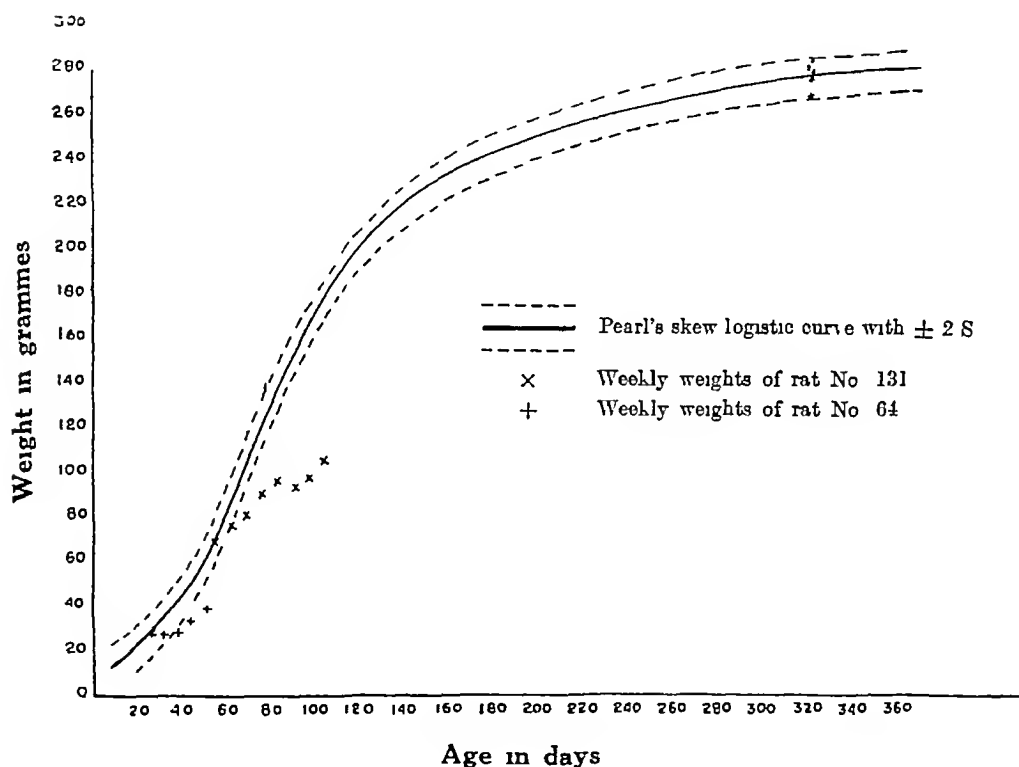


as also from Donaldson's curve of growth. The period of observation of the other two female rats on pure mustard oil is not sufficiently long to permit of a curve being calculated but the general trend appears to be consistent with logistic curve though materially different from Donaldson's. In the case of male rats the number

of observations are too small but even when they are fairly large as in rat No 131 the growth rate does not satisfy the law of logistic growth. This is probably due to some unknown circumstances depressing its growth in the 6th week, and although no definite statement can be made the general trend in both cases appears to follow

GRAPH 6

Growth of male rats fed on pure mustard oil

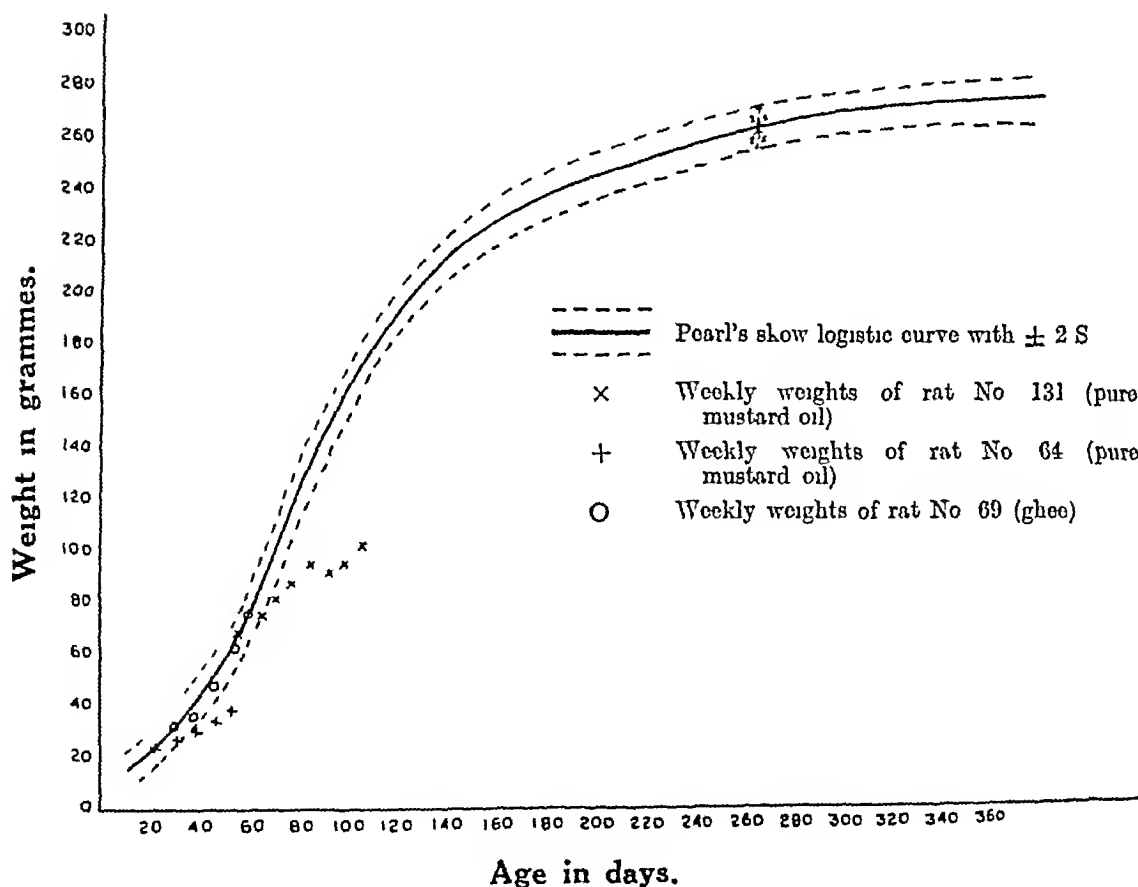


more or less the logistic curve. Here again the rate of growth is depressed as compared with that of Donaldson's rats. So also is the case with the 4 female rats just considered. The question arises whether the mustard oil depresses the rate of growth of the rodents or the observed lower rate of growth is wholly or

partly due to the unsuitability of the basic diet or of the environmental conditions. A partial answer to this question is provided by a perusal of Graphs 2 and 7 for it is seen that both in the case of female and male rats the curves of growth for ghee treated rats follow closely those of Donaldson's rats. In the case of males the observed points remain well within the range of plus or minus twice standard error

GRAPH 7

Growth of a male rat fed on ghee

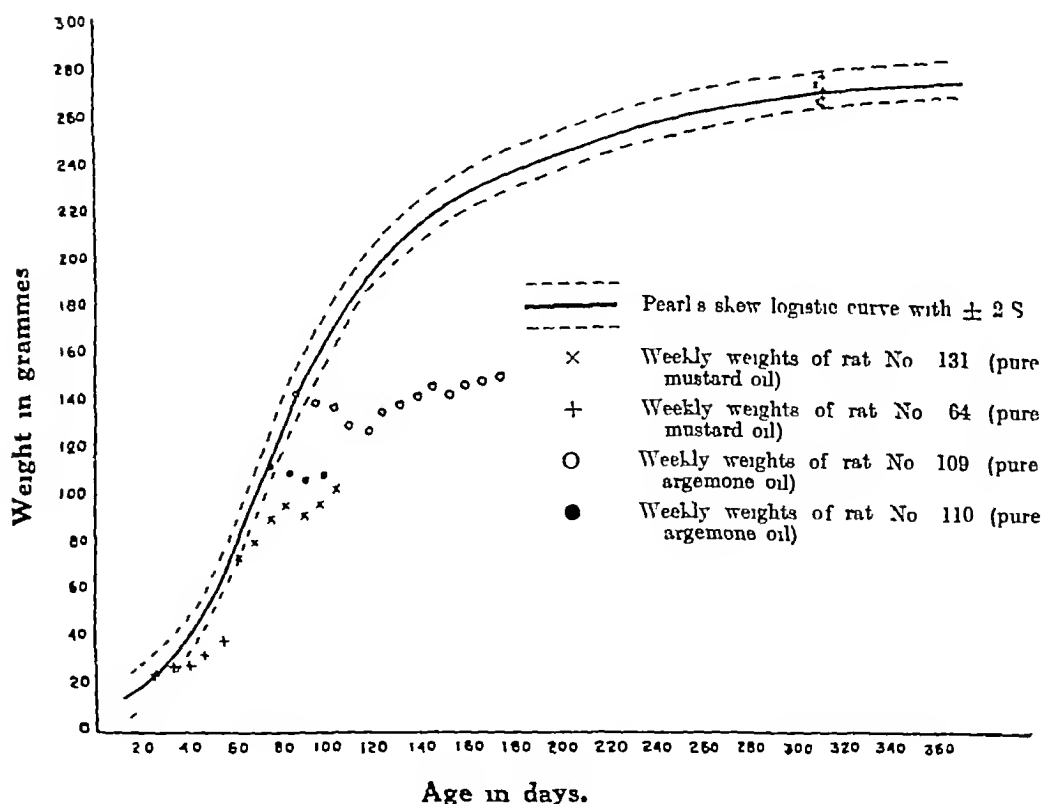


thus suggesting that the depression of growth curve in the case of mustard oil treated rats could not be due to the environmental conditions. However, the ghee treated rats were not followed for a sufficiently long period to justify calculations of their curve of growth mathematically.

Graph 8 which shows the age-weight curve of pure argemone oil treated male rats presents quite a different picture. Almost immediately after the commencement of the experimental diet the animals depart from the expected curve of growth and though still in the logarithmic phase of growth on account of age they show a

GRAPH 8.

Growth of male rats fed on argemone oil (pure)

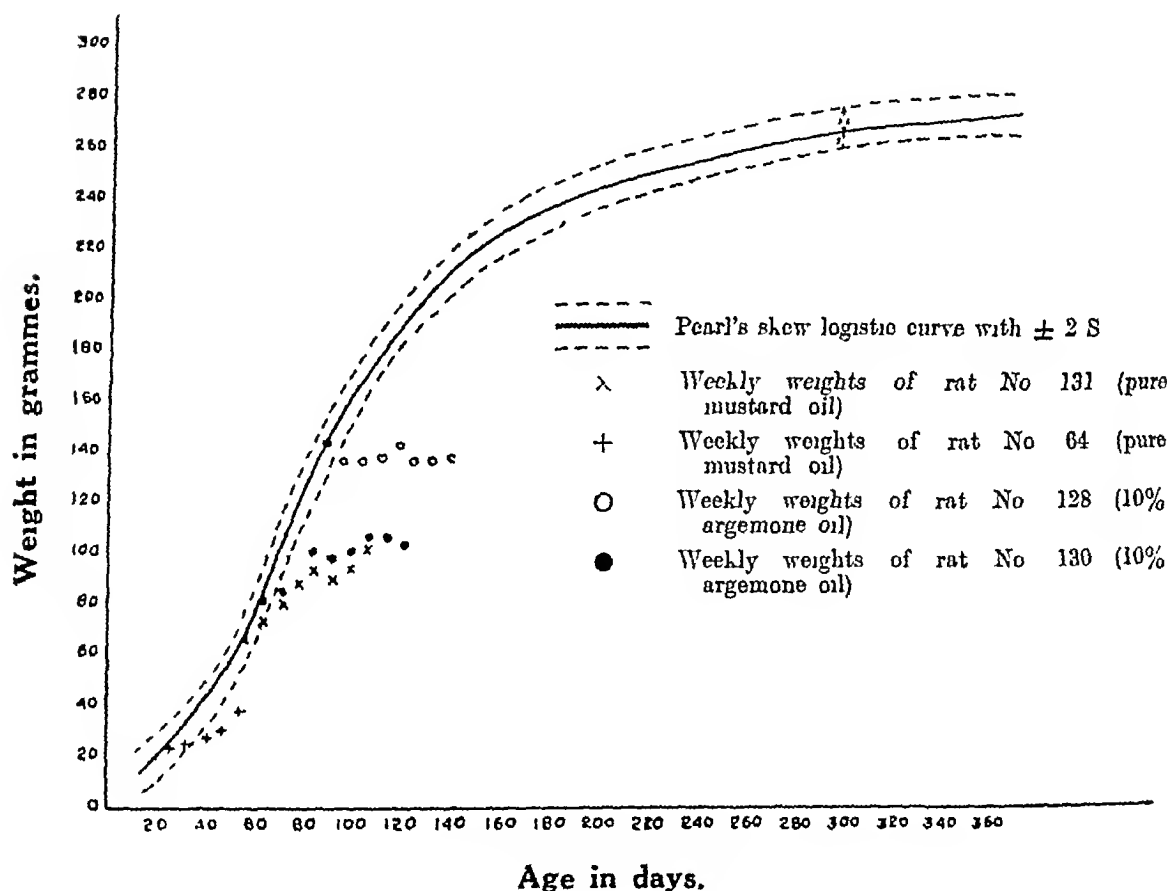


loss in weight. The curves are in fact obviously different from Pearl's curve as also from the curve of mustard oil treated rats. The same remarks apply to the female and male rats put on 10 per cent argemone oil and on Rangpur oil (*vide* Graphs 3, 4, 9 and 10).

In the case of 'suspected oil' (*vide* Graphs 5 and 11), the results cannot be definitely interpreted because it is not possible to differentiate the curves of growth of these rats from those of rats put on pure mustard oil. If it is permissible to accept the view that the marked depression of growth shown by rats put on argemone

GRAPH 9.

Growth of male rats fed on 10 per cent argemone oil in mustard oil



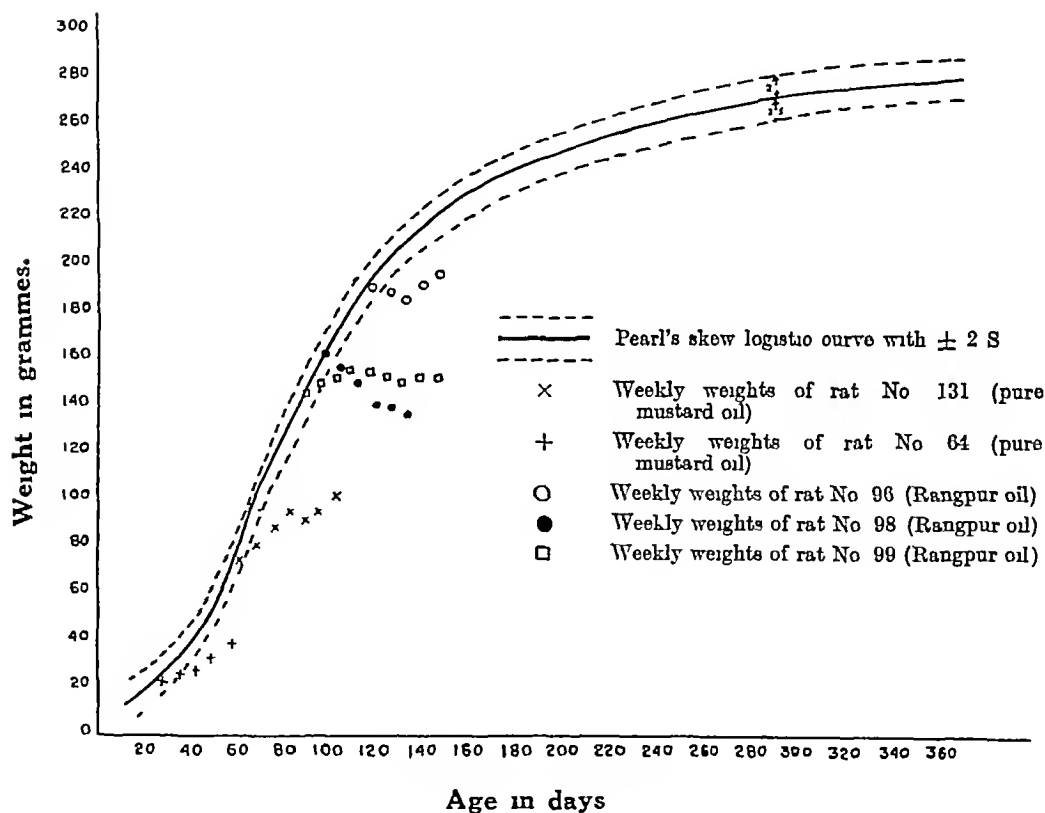
or Rangpur oil is an evidence of toxicity of these oils then it may be stated that the toxicity of the 'suspected oil' remains unproved

Pathological changes—The depression in the rate of growth, however, does not prove the specificity of toxic property of oil in relation to epidemic dropsy and,

therefore, this test by itself though highly suggestive cannot suffice for detection of specific toxicity. As has been noted previously the animals do not exhibit any signs or symptoms comparable to the disease as seen in man. One way of approach to the problem is to see whether the rodents put on argemone oil or oil proved

GRAPH 10

Growth of male rats fed on Rangpur mustard oil



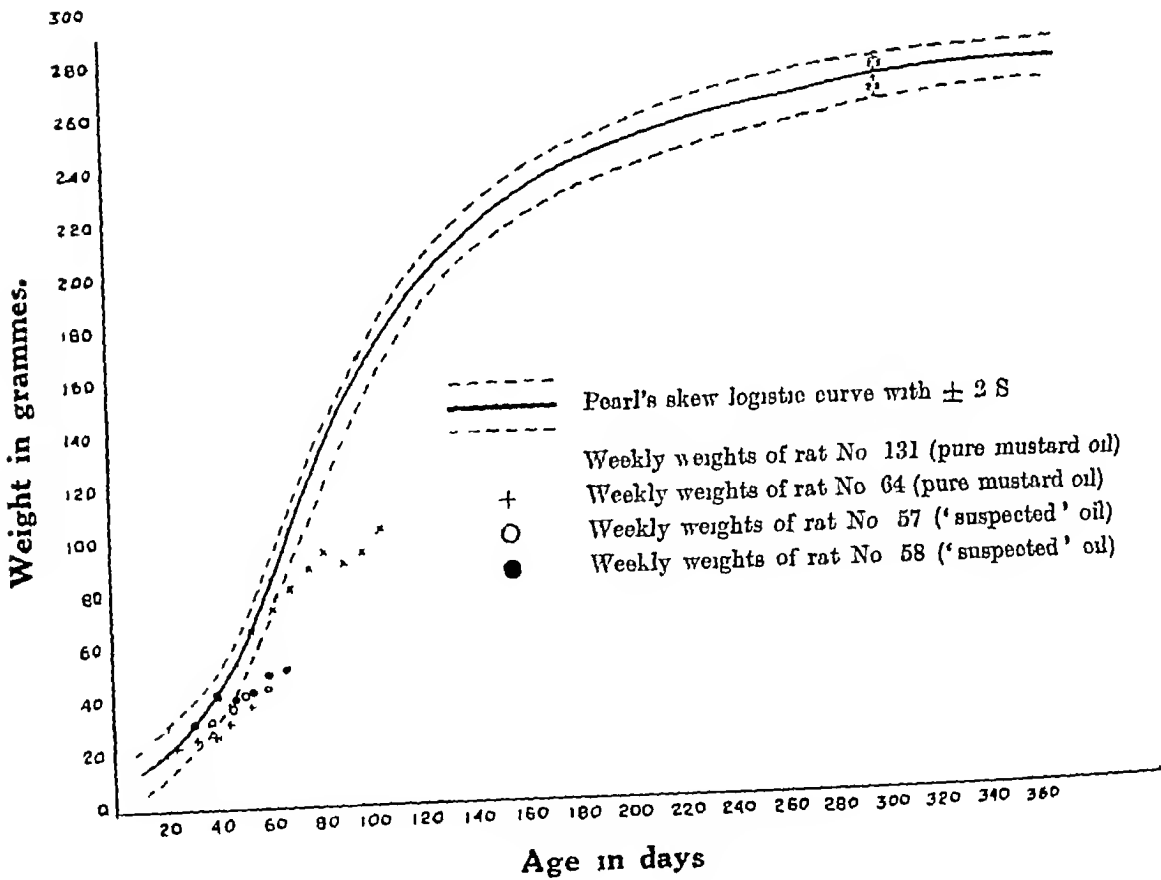
positive for the specific toxic substance by human experiment, could induce pathological changes in tissues simulating those seen in patients of epidemic dropsy

The essential histological changes induced by the disease in the various tissues and organs of man consist of the appearance of a large number of young and dilated

capillaries One of the characteristics of the young capillaries is that they are lined with active endothelial cells containing a considerable amount of protoplasm, both the nucleus and protoplasm taking up deep stain Besides, there is a great deal of proliferation of endothelial cells around the dilated vessels The pathological changes are readily seen in the skin (Lal and Das Gupta, 1941) According

GRAPH 11.

Growth of male rats fed on 'suspected' mustard oil



to Chopra, Chaudhuri and Panja (1935), in addition to changes already mentioned, there is papillary and subpapillary oedema resulting in flattening out of the papillae In the subcutaneous tissue there is evidence of oedema as shown by the looseness of the connective tissue fibre and increase in the oedematous collagenic fibres

The normal rat skin (*vide* Plate V, fig 1) resembles the human skin generally, although the appearance is greatly modified due to larger number of hair follicles and the smaller thickness of the various layers, particularly of the *Stratum corneum*. However, the main features of histology with which we are concerned, e.g. the non-patency of capillaries and the frequency with which they are seen in skin, are common to both man and rat.

Neither the record of changes in the capillaries nor the extent of tissue changes observed in individual rats used in the experiment can be adequately represented in a tabular form. A better idea may be obtained from the microphotographs of the sections of skin and other organs (*vide* Plates V and VI). A brief description of the observed changes is given below —

Rats fed on pure mustard oil—Skin snips were taken at approximately fortnightly intervals.

Of the 3 rats (Nos 131, 132 and 133) 2 (Nos 131 and 133) showed very few dilated and engorged papillary blood vessels in the skin after 20 days of feeding. After 54 days' feeding some increase in the collagenic fibres was observed in skin sections of all the 3 rats, but there was no evidence of vascularization. The same was true of skin section of one of the rats fed for 70 days (*vide* Plate V, fig 2). No signs of oedema were noticed. Vessels of heart, lungs and kidneys showed engorgement in all the animals.

Rats fed on argemone oil—Typical pathological picture of advanced changes effected in the skin of rat by ingestion of toxic oil were demonstrated in rat No 109, 94 days after the commencement of experiment (*vide* Plate V, fig 3). A large number of highly dilated capillaries engorged with blood is seen in the dermis. There is perivascular infiltration of endothelial cells. The collagenic fibres are thickened. Rat No 110 which died of congestion of lungs on the 33rd day of experiment did not present demonstrative histological changes.

Rats fed on 10 per cent argemone oil in mustard oil—Rats Nos 128, 129 and 130 received this oil. Snips of skin were taken at about fortnightly intervals and the animals were stunned to death after 51, 54 and 55 days respectively. In No 128, a large number of dilated capillaries was observed in the dermis of snips taken on the 51st day but not in the ones taken prior to that day. Heart, lungs, kidneys and intestine were found congested. In the series of skin sections of rats Nos 129 and 130 more marked progressive changes were observed than in No 128. Thus, in rat No 130 while in the skin snips taken on the 19th day except for increase in collagenic fibres no definite changes could be demonstrated, in the one taken on the 33rd day marked increase in the number of dilated capillaries and cellular infiltration was observed (*vide* Plate VI, figs 5 and 6). More marked changes were seen in the section taken on the 55th day (Plate VI, fig 7). A large number of new capillaries was observed in the heart of No 129 (*vide* Plate VI, fig 8). Most of the internal organs of Nos 129 and 130 showed congestion.

Rats fed on Rangpur oil—In this group animals were killed at periodic intervals to find out if any progressive histological changes were taking place. In animals

Nos 95 and 96, killed on the 16th and 26th days, no changes were observed in the histology of skin but congestion was found in internal organs. In animals Nos 99 and 100, killed on the 60th day, there was considerable increase in the number of dilated capillaries in the *Stratum corneum* together with perivascular infiltration of endothelial cells. Changes were similar to those seen in rat No 109. Masses of endothelial cells were also observed scattered about the *dermis* in close relation to capillaries. Collagenic fibres in the connective tissue of the skin were greatly increased (*vide* Plate V, fig 4). No sign of œdema was, however, noticed. While these changes were quite marked in these animals, rats Nos 97 and 98 which were killed earlier (29th and 40th days) also showed similar changes but in lesser degree, thus suggesting that the changes were progressive.

The above description and a perusal of the figures in Plates V, VI and VII brings out the following points —

There is a considerable amount of vascularization of the skin and of internal organs in the rats fed on oil containing argemone oil or samples of mustard oil giving the reactions for this oil. However, the control group, namely the rats fed on mustard oil, also shows dilatation of vessels in skin, but the difference between the two series is mainly in regard to amount of vascularization. In the group of rats fed on toxic oils there is considerable increase in the number of young capillaries and in perivascular infiltration of endothelial cells.

Changes in the histological structure of skin in the toxic oil series are characteristic and closely resemble those induced by the disease in man. These changes are progressive in nature as may be seen from Plate VI, in animals in which the biopsies were carried out at roughly 15 days' intervals. There is, however, one important difference between rat and man, namely the absence of œdema in the former case.

Thus, it is seen that young rats may be used as test animals for 'suspected mustard oil' and for the suspected compounds derived from such oil.

EXPERIMENTS WITH OTHER ANIMALS

Cats — A series of experiments was conducted on cats. In this case besides pure mustard oil, olive oil was also used as control and 5 per cent argemone oil in pure mustard oil was employed as a check. No experiment was performed with the suspected oil referred to above but Rangpur oil was used.

Mortality — Of 8 cats put on pure mustard oil only 2 died early, namely on the 11th and 35th days, the rest with one exception survived till the end of the experiments which lasted from 65 to 120 days. The one that died on the 11th day rapidly lost weight from the very beginning and on post-mortem it was found to have pneumonic patches in the base of lungs.

None of the 2 olive oil cats died till the end of the experiment.

Of the argemone oil group both the cats died, one on the 34th day and the other on the 74th day.

PLATE V.



Fig 1—Skin of normal rat



Fig 2—Skin of a rat fed on pure mustard oil for 70 days



Fig 3—Skin of rat (No 109) fed on pure argemone oil for 94 days



Fig 4—Skin of rat (No 100) fed on Rangpur oil for 59 days

PLATE VI

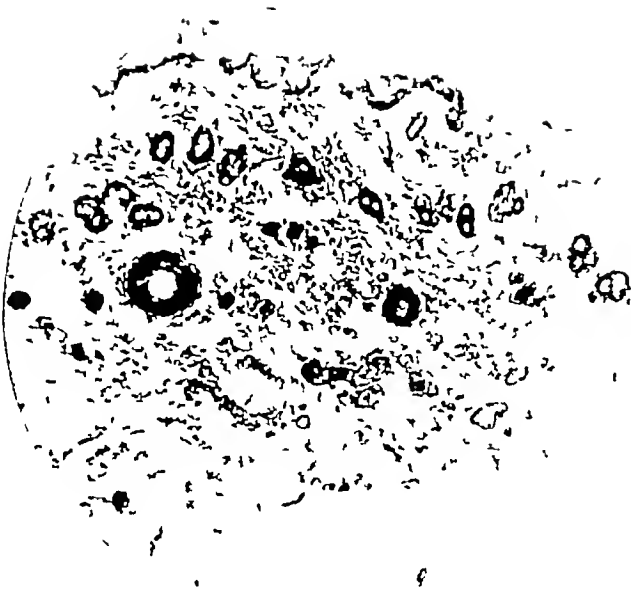


Fig 5 —Skin of rat (No 130) fed on argemone oil (10 per cent) for 33 days



Fig 6 —Same section as in Fig 5 under higher magnification



Fig 7 —Skin of rat (No 130) fed on argemone oil (10 per cent) for 54 days Note the large number of dilated capillaries



Fig 8 —Heart of rat (No 129) fed on argemone oil (10 per cent) for 54 days



Fig 9 —Skin of normal cat

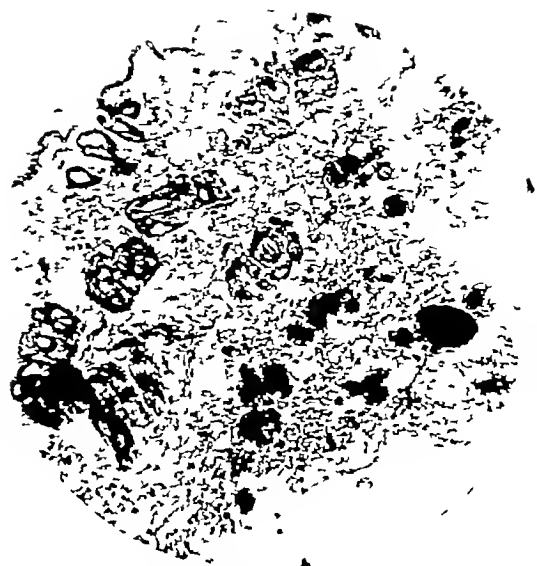


Fig 10 —Skin of cat (No 124) fed on pure mustard oil for 65 days



Fig 11 —Skin of cat (No 116) fed for 52 days



Fig 12 —Skin of cat (No 116) fed on argemone oil (5 per cent) for 70 days. Note the masses of capillaries in the deeper layers

Of 5 per cent argemone oil group one was killed on the 59th day, the other on the 70th day. In the Rangpur oil group there was heavy mortality, 5 out of the 6 cats having died, 3 within 21 days and the other 2 on the 40th and 43rd days. The general impression one got from these records was that on the whole the mortality of the cats put on toxic oils took place earlier than of those put on mustard oil, especially if we were to exclude the animals that died very early presumably from some intercurrent disease. However, this by itself cannot be taken as a test of toxicity because one of the cats on pure argemone oil survived as long as the 74th day.

Changes in weight—Unfortunately we have no basic growth curve of cats as we have of rats and therefore it is difficult to come to a definite conclusion from study of the weight records. It may, however, be stated that all the animals used in the experiment were young although their exact age was not known. However* a study of the average gain or loss in weight per week brought out two obvious conclusions, namely (1) except in the case of the cat No 105 which died very early all other animals put on mustard oil gained weight which was considerable in most instances. So was also the case with cats put on olive oil. On the other hand, the cats put on one or the other toxic oil showed only a nominal increase in weight or an actual loss. The Rangpur oil cats' were better off in this respect than those on 5 per cent pure argemone oil.

Pathological changes—Here also the same type of changes in histology of skin was observed as in the rats but with this difference that while in the beginning progressive vascular changes were observed, later on the vessels, instead of showing active lining cells, exhibited atrophy of these cells and instead of remaining engorged with blood they were empty. Histological changes are briefly summarized below.

Pure mustard oil group—Sections of skin and organs of animals Nos 89, 90, 105, 106, 123 and 124 were examined. Congestion of some of the internal organs which varied from cat to cat was observed. No change was observed in the skin section in any of the animals (*vide* Plate VII, fig 10). For purposes of comparison skin section of normal cat is shown in Fig 9.

Argemone oil group—Collagenic fibres in connective tissue of skin were greatly increased in both Nos 113 and 114. No changes in the vascularity of the skin were observed. Internal organs in No 113 were found congested. It may be mentioned in this connection that these animals did not retain the food well.

Five per cent argemone oil—In both the animals Nos 115 and 116 a large number of capillaries was seen in the corium of skin after 50 days of experimental feeding. Perivascular infiltration of endothelial cells was noticed. Masses of endothelial cells were found scattered in the corium and small capillaries were seen in their midst. The collagenic fibres were increased. These changes were

* The actual data have been omitted to save space. Details can be supplied to those interested in them.

progressive as shown by skin sections taken at intervals during the course of the experiment (*vide* Plate VII, figs 11 and 12) Internal organs were not much affected except the liver which was found congested in both animals

Rangpur oil—Skin and most of the organs were not demonstrably affected Heart showed congestion in both the cats, while lungs in No 110 and liver and kidneys in No 103 were congested

Guinea-pigs—This animal does not appear to be suitable for the test because it did not flourish very well on pure mustard oil Of the 6 animals in this group 3 died and all of them lost weight considerably No experiment was conducted with pure argemone oil or known mixture of argemone oil in mustard oil but experiments were conducted with Rangpur oil and with two other suspected oils, one of which (Alamdanga oil) was found toxic on human experiment, the other (S L oil) was highly suspicious on epidemiological grounds Congestion of internal organs was observed in all the guinea-pigs irrespective of the type of oil given Skin showed no noticeable changes

Pigs—Two very young pigs were employed, one being put on Rangpur oil, the other on pure mustard oil These animals survived 65 days of experimentation Both gained considerable weight, the one on pure mustard oil having added relatively a little more weight than the other Nothing of consequence was found on post-mortem nor on histological examination of tissues except congestion of kidneys in both the animals

SUMMARY

1 Feeding experiments have been carried out on a number of animals, namely rats, cats, guinea-pigs and pigs, with a view to finding out a suitable biological test for the detection of specific toxicity in respect of epidemic dropsy in samples of suspected mustard oil Three criteria have been taken into account, namely time to death, loss of weight and the characteristic histological changes in the tissue, particularly the skin

2 Different hatches of rats (*Mus norvegicus albinus*) were fed on ghee, on pure mustard oil, on pure argemone oil, on 10 per cent argemone oil in mustard oil, on a known toxic oil (Rangpur oil) and on a suspected mustard oil obtained from the field In the absence of knowledge of exact age of the rodents and of the growth curves it has been assumed that prior to the experiment the female rats were subjected to the growth curves described by Donaldson, and the male rats to the skew logistic curve devised by Pearl on Donaldson's data The growth curves of rats in different groups were then compared with one another and with the assumed normal curves of growth

3 Comparisons of the periods indicating time to death in the course of experiments were made to estimate the toxic influence of the samples The specificity of toxic properties of the oil samples was estimated by the appearance

of pathological changes simulating those observed in patients of epidemic dropsy in the skin and other organs of test animals. The progressive nature of the tissue changes was observed in post-mortem material of animals killed at intervals or in skin snips obtained in biopsies at regular intervals.

4 Groups of cats were fed on pure mustard oil, on olive oil, on pure argemone oil, on 5 per cent argemone oil and on a known toxic oil (Rangpur oil). Points investigated were similar to those in rats, namely loss in weight, time to death, and histological changes in skin and other organs. No growth curves for normal cats were available as in the case of rats but marked differences in weight of these animals put on different diets were obtained. Similar investigations were carried out on guinea-pigs and pigs. The former were fed on Rangpur oil and on two suspected oil samples. Neither of these animals appeared to be suitable for the biological test.

CONCLUSIONS

1 Clinical signs of epidemic dropsy could not be induced in rats, cats, guinea-pigs or pigs.

2 Rats and cats but not guinea-pigs and pigs are suitable to serve as test animals for suspected oil samples. The rats are particularly suitable on account of the ease with which they can be handled, besides, they provide a more delicate test of toxicity and consume smaller amounts of the oil to be tested.

3 Addition of pure mustard oil to the basic diet depresses growth, the growth curves of the rats put on this material, although consistent with the logistic law, are materially different and lower than the standard curves. The rodents put on ghee (clarified butter) instead of oil followed the standard curves of growth, thus suggesting that the depression of growth in the case of mustard oil rats was not due to influences arising from the environmental conditions of the experiment.

Addition of pure argemone oil or 10 per cent argemone oil in mustard oil or of epidemiologically incriminated and experimentally proved toxic oil (Rangpur oil) to the standard diet causes still greater depression of growth curve which no longer follows the logistic law. Depression in the rate of growth is also noticed in cats fed on toxic oils.

4 Histological changes in the skin of rats fed on pure argemone oil or on a mixture of argemone oil in mustard oil and the toxic mustard oil (Rangpur oil) resemble those found in the skin of patients of epidemic dropsy. Thus, in the dermal layer is found a large number of dilated capillaries which are absent in the normal animals as also in those fed on pure mustard oil. There is perivascular infiltration of endothelial cells. The collagen bundles are increased. Similar changes may be observed also in heart muscles in certain cases.

Cats fed on toxic oil also show similar changes except that in the later stages of the experiment the vascular walls are collapsed and do not exhibit the characteristic young endothelial cells.

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APPENDIX

It has been stated in the body of the paper that the main arguments are clearly indicated in the graphs and require no elaborate statistical discussion. However, it has been considered desirable to set out the statistical method of approach and the line of statistical arguments developed in the treatment of these data so that those interested in this aspect of the problem may be able to follow the subject in greater detail.

Theoretically the problem is to compare the observed weekly weights of rats put on different treatments with the corresponding expected values for normal rats put on the basic diet. This comparison could be made in two stages: (1) to test whether the observed data follow the logistic law and (2) in case they do, to compare the curve of growth fitted to the observed data with the curve of growth fitted to the data for rats on basic diet.

For male rats Pearl (*loc cit*) has fitted a skew logistic curve on Donaldson's data, the equation to the curve being—

$$y = 7 + \frac{273}{1 + e^{4.3204 - 7.2196x + 30.0878x^2 - 0.5291x^3}}$$

where y is the weight in grammes and x the age in 100-day units.

For female rats we have no such standard curve. The only curve of growth available for the purpose is that given by Donaldson (*loc cit*). This curve is made up of two different curves, one a simple parabola—

$$y = 8.071 + 0.367x + 0.0131x^2$$

for the age period between 10 and 80 days, the other a logarithmic curve—

$$y = 343 \log x - 0.41x - 498.8$$

for the age period 80 and 365 days.

Only in one instance, namely rat No. 109, the experimental period is long enough for fitting a skew logistic curve and for comparing the fitted curve with Pearl's skew logistic curve. However, it is apparent from the graphs that the growth curve in this case does not follow a skew logistic curve. The curve has therefore not been fitted.

Including rat No. 109, there are 9 rats in all, 5 males and 4 females, for which the simple logistic hypothesis could be tested. To test the hypothesis of the simple logistic curve two methods are available—

- (1) The direct method. After fitting the curve the goodness of fit may be tested by the method of analysis of variance. This method requires a knowledge of the age of the rats at the commencement of the experiment. We did not have this knowledge.
- (2) The second method consists in fitting a straight line to relative growth rates against weights which Fisher (1936) has shown to be equivalent to fitting a simple logistic curve to weights against time. Thus, if

w be the weight of a rat at time t , the differential equation of the simple logistic curve is—

$$\frac{1}{w} \frac{dw}{dt} = a - bw$$

where a and b are two positive constants

Now, $\frac{1}{w} \frac{dw}{dt}$ ($= \frac{d}{dt} \log_e w$) is the same as the relative growth rate,

and if we put $y = \frac{1}{w} \frac{dw}{dt}$, the equation becomes $y = a - bw$ which is the equation of a straight line. This transformation of a complicated equation into a straight line simplifies the test for significance and is, therefore, a great advantage. This method is particularly apt in the present case because a knowledge of the actual initial age of the rats is not required.

In order that a straight line may be a good fit to relative growth rate against weight two conditions are to be satisfied. The first is that the 'sums of squares' due to the straight line regression must be significant as against the sums of squares due to deviations from the straight line regression. This is a necessary condition, but not a sufficient one, i.e. if this condition is not satisfied the hypothesis of the straight line fit falls and there is no need to proceed further. When this condition is satisfied we are still not in a position to say that the simple straight line is a reasonably good fit to the data. To be able to say this we have to proceed a step further and to test that the reduction in the sum of squares due to deviations from regression when a parabola is fitted instead of a straight line is not significant. Thus the second condition to be satisfied, after the linear regression function has been shown to be significant, is that the reduction in the sum of squares due to deviations is not significant when a second degree parabola is fitted.

The relative growth rates and the corresponding weights for each rat are set out in Table I —

TABLE I

The relative growth rates of rats against weekly weights

Rat number	Sex	Weekly weights (g)	Relative growth rates (per cent per day)	Rat number	Sex	Weekly weights (g)	Relative growth rates (per cent per day)
99	♂	150	0.47626	100	♀	127	-0.50429
		155	0.23422			123	-0.34557
		155	0.00000			121	-0.17500
		155	-0.09372			120	-0.30000
		153	-0.18665			116	-0.05977
		151	0.04650			119	0.24207
		154	0.14043			120	0.11907

* The geometrical representation of $\frac{1}{w} \frac{dw}{dt}$ is given by G. U. Yule, in his Presidential Address to the Royal Statistical Society, 1925.

TABLE I—*concl'd*

Rat number	Sex	Weekly weights (g)	Relative growth rates (per cent per day)	Rat number	Sex	Weekly weights (g)	Relative growth rates (per cent per day)
109	♂	140	—0 25429	130	♂	85	0 57829
		138	—0 52943			90	1 30229
		130	—0 53736			102	0 68098
		128	0 26957			99	0 14126
		135	0 64014			103	0 69279
		140	0 36114			108	0 40443
		142	0 39686			109	—0 13336
		148	0 09986	131	♂	75	1 35557
		144	0 00000			81	1 30229
		148	0 29157			90	1 21343
128	♂	150	0 19043			96	0 15707
						92	0 07407
						97	0 94407
				132	♀	94	1 56050
						107	1 47386
						118	1 11085
						125	0 52500
						127	0 22500
						129	0 43636
						135	0 41036
129	♀	138	—0 35343	133	♀	91	0 64536
		138	0 10278			94	0 81507
		140	0 30393			102	0 44203
		144	—0 05136			100	0 06979
		139	—0 25250			103	0 07107
		139	0 10215			101	0 06886
						104	0 14015
		104	—0 26465				
		103	0 07557				
		105	0 13729				
		105	—0 20707				
		102	—0 20707				
		102	0 20707				
		105	0 27485				

In Table II are presented the analyses for the 9 rats mentioned above. The methods of working out this table from the data given in Table I will be found in any standard textbook on statistics.

TABLE II

Analysis of variance of relative growth rates of rats put on different treatments

Rat number	Sex	Source of variation	Sum of squares	Degrees of freedom	Variance	F *	5 per cent point for F	REMARKS
99	♂	Due to linear regression	0 06400	1	0 06400	1 40	6 61	The hypothesis of logistic curve falls through
		Due to deviations from linear regression	0 22843	5	0 04569			
		TOTAL	0 29243	6				
100	♀	Due to linear regression	0 21062	1	0 21062	5 00	6 61	The logistic curve hypothesis is not tenable
		Due to deviations from linear regression	0 21044	5	0 04209			
		TOTAL	0 42106	6				
109	♂	Due to linear regression	0 05973	1	0 05973	0 40	5 12	The logistic curve hypothesis is untenable
		Due to deviations from linear regression	1 35244	9	0 15027			
		TOTAL	1 41217	10				
128	♂	Due to linear regression	0 00630	1	0 00630	0 09	7 71	The hypothesis of logistic curve is not tenable
		Due to deviations from linear regression	0 29470	4	0 07368			
		TOTAL	0 30100	5				

* F = ratio of the variance with lesser number of degrees of freedom to the variance with greater number of degrees of freedom

TABLE II—*contd*

Rat number	Sex	Source of variation	Sum of squares	Degrees of freedom	Variance	F *	5 per cent point for F	REMARKS
129	♀	Due to linear regression.	0 00498	1	0 00498	0 09	6 61	The logistic curve hypothesis is untenable
		Due to deviations from linear regression.	0 29376	5	0 05875			
		TOTAL	1 81638	6				
130	♂	Due to linear regression	0 41584	1	0 41584	2 48	6 61	The logistic curve hypothesis is not tenable
		Due to deviations from linear regression	0 83887	5	0 16777			
		TOTAL	1 25471	6				
131	♂	Due to linear regression.	0 73958	1	0 73958	3 14	7 71	The logistic curve hypothesis is not tenable
		Due to deviations from linear regression	0 94326	4	0 23582			
		TOTAL	1 68284	5				
132	♀	Due to linear regression	1 57055	1	1 57055	31 94	6 61	The regression function is highly significant
		Due to deviations from linear regression	0 24583	5	0 04917			
		TOTAL	1 81638	6				

* F = ratio of the variance with lesser number of degree of freedom to the variance with greater number of degrees of freedom

TABLE II—concl'd

Rat number	Sex	Source of variation	Sum of squares	Degrees of freedom	Variance	F *	5 per cent point for F	REMARKS
133	♀	Due to deviations from linear regression	0.24583	5	0.05946			The reduction in the sum of squares is not significant. Hence the hypothesis is not disproved.
		Due to deviations from parabolic regression	0.23785	4				
		DIFFERENCE	0.00798	1	0.00798	0.13	7.71	
		Due to linear regression	0.37913	1	0.37913	9.17	6.61	The regression function is significant.
		Due to deviations from linear regression	0.20668	5	0.04134			
		TOTAL	0.58581	6				
		Due to linear regression	0.20668	5				The parabola is definitely worse fit than the straight line as the deviation from parabola is greater than that from the straight line. Hence the hypothesis of growth according to the logistic law is tenable.
		Due to deviations from parabolic regression	0.20856	4				
		DIFFERENCE	-0.00188	1				

* F = ratio of the variance with lesser number of degrees of freedom to the variance with greater number of degrees of freedom

Thus, we see that in none of the 5 male rats the growth according to the logistic law is tenable. But of the 4 female rats 2, both fed on pure mustard oil, do not disprove the hypothesis. However, as will be seen below, although the 2 rats follow logistic law the curves are significantly different from each other. The equation of the two straight lines fitted by the method of 'Least squares' to relative growth rate against weekly weights are—

$$Y = 5.28300 - 0.03731x$$

$$\text{and } Y = 5.43472 - 0.05149x$$

Y indicates the relative growth and x the weight for rats Nos 132 and 133 respectively.

The variance of the regression coefficient of rat No 133 ($= 0.00144$) is almost seven times the variance of the regression coefficient of rat No 132 ($= 0.00022$) and this difference is significant and hence the respective logistic curves differ significantly from one another. The equations to the two logistic curves are —

$$Y = \frac{141.6}{1 + 20.049 e^{-0.05283t}} \text{ for rat No 132}$$

$$\text{and } Y = \frac{105.6}{1 + 7.801 e^{-0.02360t}} \text{ for rat No 133}$$

Y here being the weight and t the time

Before closing it may be mentioned that Donaldson's curve for female rats has not been fitted to the observations of female rats under experiment for two reasons. Firstly, the graphs in the body of the paper clearly show that none of the 5 female rats can be said to follow the Donaldson's curve as the deviations from Donaldson's curve are obvious. Secondly, even if a logarithmic curve of the Donaldson's type—not the Donaldson curve but a similar curve with different constants—was found to fit the observations, we had no means of testing the significance of the difference.

URINARY EXCRETION OF MORPHINE IN OPIUM ADDICTS WITH AND WITHOUT LECITHIN-GLUCOSE TREATMENT

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CHOPRA AND ROY (1937) made a study of the cholesterol and lecithin content of the blood of opium addicts during addiction, after discontinuation of opium, and also after a course of treatment with lecithin. They found more or less normal values for these constituents in the different stages excepting that there was an increase of the lecithin content during and after the course of lecithin treatment and in the majority of cases during the period of abstinence as well. They also found that morphine could be detected in the urine for 4 or 5 days after the taking of the last dose. The present paper is a continuation of the former one and embodies the results of further work on this subject to see if the lecithin-glucose treatment so successfully employed by Chopra and Chopra (1940) in relieving the withdrawal syndrome has any appreciable effect on the rate of excretion of morphine.

The examination of the urine of opium addicts for morphine content has now become almost a routine procedure for all the cases that are treated in the Carmichael Hospital for Tropical Diseases, firstly to confirm the diagnosis of opium addiction, secondly to determine the effects of treatment and also to see whether the addicts are taking the drug clandestinely while they are undergoing

treatment in the hospital. The last named point is very important from the point of view of treatment as during withdrawal the craving for the drug becomes so great that the addict sometimes procures the drug by some means and takes it surreptitiously, all the time maintaining that he has been observing the strictest abstinence, thus counteracting the effects of treatment. The examination of the urine, however, reveals the real state of affairs and this enables the physician to take proper steps in time.

The method employed for the detection and estimation of morphine is a slight modification of that employed by Decarté (1937).

Ten c.c. of the urine are heated with about 0.3 g. of Na_2CO_3 until the first bubbles rise and then quickly cooled. It is then transferred to a glass stoppered 25 c.c. cylinder and thoroughly shaken with 10 c.c. of acetic ether. After the layers have separated, the acetic ether layer is transferred to a small porcelain dish and evaporated to dryness on a water-bath. To the residue are added 0.25 c.c. of distilled water, one drop of nitric acid (sp. gr. 1.15) and a drop of 10 per cent ammonium molybdate solution. After careful whirling of the porcelain dish, the contents are filtered through a small filter paper. The dish is rinsed with two successive small quantities of water (0.2 c.c.) and passed through the filter. A drop of 2 per cent solution of ammonium vanadate is then added and well mixed. A turbidity develops, depending on the morphine content, which is matched against a set of standard tubes of uniform bore containing varying amounts of morphine and to which the same quantities of nitric acid and ammonium vanadate have been added, after adjustment of the unknown and the standard to the same volume say 0.8 c.c. This method can detect morphine of the order of 0.005 mg. and may be used as a quantitative procedure when very great accuracy is not intended.

In order to see whether this procedure can be applied for the correct diagnosis of opium addiction, it was tried on a certain number of urine samples collected from persons of normal health and other patients in the hospital who were not opium addicts. All these samples gave negative results. This test therefore affords a fairly reliable means for the detection of morphine in the urine of opium addicts.

As the drug is largely used in India and as cases from the remotest corners of the country, where there are no hospitals or laboratories and where transportation is difficult, might have to be diagnosed, experiments were carried out to ascertain if delay in the examination of samples of urine affected the results in any appreciable manner. A certain number of urine samples tested for morphine content and then kept for varying periods from 2 to 7 days at the ordinary temperatures of the laboratory was again tested for morphine content afterwards. All these samples gave positive results and the quantity of morphine was found to have suffered little deterioration on keeping. These observations are of interest from a medico-legal standpoint as it appears possible to detect morphine in the dead body even after decomposition has set in. As the drug is very rapidly eliminated, and as it is not usually detected in the urine 4 or 5 days after the taking of the last dose for diagnostic and other purposes, the urine should be collected as early as possible though the actual test may be delayed for some days.

For the purpose of the present investigation addicts were studied under two heads —

Group I consisted of cases who received lecithin and glucose immediately after withdrawal of the drug. Group II consisted of those who did not receive any lecithin either by mouth or by injection.

The results are shown in Tables I and II. Table III is only an abridged form of Tables I and II and indicates what percentage of the cases show the presence of morphine in the urine on a particular day after withdrawal.

It would appear from Tables I and II that the amount of morphine excreted bears no relation to the amount of daily dose taken or to the duration of addiction. Sometimes, with a comparatively smaller dose, the amount excreted through the urine may be very significant, on the other hand when a much bigger dose is continued for a longer period, the quantity of morphine excreted may not be appreciable even during the first few days. This is in accordance with the observations of Chopra and Roy (*loc cit*). Another significant fact is the relatively poorer excretion rate of morphine when the drug is smoked than when taken per mouth in the form of pills.

A perusal of Table III will show that morphine could be detected in the urine of addicts who had not had any treatment, for a longer period than those treated with lecithin and glucose or, in other words, treatment with lecithin and glucose aids in effecting a more rapid elimination of morphine in the urine. For example, on the first day after withdrawal all the cases in both sets showed presence of morphine in the urine. On subsequent days a smaller percentage of the treated cases showed the presence of morphine in their urine than those having no treatment. Of the treated cases none showed the presence of morphine in the urine after the 6th or 7th day but, in the non-treated cases, morphine could be detected in the urine even up to the 10th day. The quicker elimination of morphine in the treated cases may probably be due to the diuresis caused by glucose.

SUMMARY

- 1 The rate of excretion of morphine in the urine of opium addicts, some of whom were treated with lecithin and glucose, was studied.
- 2 The time taken to render the urine morphine-free appears to be shorter in the case of addicts treated with lecithin and glucose than in the untreated cases.
- 3 The rate of excretion of morphine bears no relation either to the daily dose of opium or to the duration of addiction.
- 4 The morphine content of a sample of urine is not appreciably affected on keeping it for several days.

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TABLE I

Showing effects of lecithin and glucose on the excretion of morphine in the urine of 30 treated opium addicts

Age	HISTORY IN BRIEF		MORPHINE CONTENT OF THE URINE IN MG				
	Duration of addiction in years	Weighed daily dose in grains	1st day	2nd day	3rd day	4th day	5th day
30	14	28.8	4.0	1.0	1.0	Trace	Trace
38	8	6.0	Trace	0	0	0	0
45	6	14.0	1.0	Trace	0	0	0
32	5	8.0	4.0	"	0	0	0
28	3	6.0	4.0	1.0	1.0	0	0
36	2	18.0	1.0	Trace	0	0	0
40	7	10.0	2.0	2.0	1.0	0	0
30	10	6.0	1.0	1.0	Trace	0	0
34	5	6.0	9.0	1.0	"	0	0
44	12	14.0	10.0	4.0	1.0	0	0
42	8	16.0	3.0	Trace	0	0	0
32	10	16.0	Trace	"	0	0	0
25	7	4.0	3.0	1.0	Trace	0	0
38	1	46.0	1.0	3.0	1.0	0	0
60	7	44.0	Trace	2.0	1.0	Trace	0
27	15	86.0	5.0	1.0	Trace	0	0
21	6	8.0	Trace	0	"	0	0
29	3	6.0	10.0	0	"	0	0
32	2	24.0	7.0	0	"	0	0
28	4	12.0	5.0	Trace	"	0	0
39	13	6.0	2.0	1.0	"	0	0
40	4	11.0	2.0	Trace	0	0	0
37	5	14.0	1.0	2.0	Trace	0	0
28	7	6.4	2.0	Trace	0	0	0

TABLE I—*conold*

Age	HISTORY IN BRIEF		MORPHINE CONTENT OF THE URINE IN MG				
	Duration of addiction in years	Weighed daily dose in grains	1st day	2nd day	3rd day	4th day	5th day
31	16	14 0	8 0	<i>Trace</i>	0	0	0
40	2	9 6	<i>Trace</i>	0	0	<i>Trace</i>	0
42	11	6 0	2 0	1 5	0	0	0
24	7	4 0	1 0	2 0	0	0	0
31	5	18 0	12 0	5 0	13	<i>Trace</i>	<i>Trace</i> present up to 7th day
37	4	1 4	4 0	2 0	<i>Trace</i>	0	<i>Trace</i>

TABLE II

Showing rate of excretion of morphine in urine after withdrawal in a series of 24 non-treated cases

Age	HISTORY IN BRIEF		MORPHINE CONTENT OF THE URINE IN MG					REMARKS
	Duration of addiction in years	Weighed daily dose in grains	1st day	2nd day	3rd day	4th day	5th day	
28	10	12 5	3 0	2 0	1 0	<i>Trace</i>	<i>Trace</i>	
29	12	16 0	1 0	2 0	1 0	0	0	
32	8	19 5	2 0	1 0	<i>Trace</i>	0	0	
45	7	120 0	10 0	<i>Trace</i>	,	0	0	
30	10	46 0	4 0	2 0	"	0	0	
21	10	23 5	2 0	1 0	"	<i>Trace</i>	<i>Trace</i>	<i>Trace</i> present till 8th day
14	8	10 0	1 0	1 0	"	0	,	<i>Trace</i> up to 7th day

TABLE II—*concl'd*

Age	HISTORY IN BRIEF		MORPHINE CONTENT OF THE URINE IN MG					REMARKS
	Duration of addiction in years	Weighed daily dose in grains	1st day	2nd day	3rd day	4th day	5th day	
50	6	14 8	3 0	2 0	1 5	<i>Trace</i>	<i>Trace</i>	Trace till 7th day
42	3	12 5	9 0	3 6	<i>Trace</i>	2 0	1 0	Trace till 10th day
41	2	10 5	7 0	7 0	4 0	1 0	<i>Trace</i>	
43	5	18 6	3 0	3 0	2 0	0	0	
24	10	26 5	<i>Trace</i>	<i>Trace</i>	0	<i>Trace</i>	<i>Trace</i>	
25	6	13 5	„	1 0	<i>Trace</i>	0	0	
24	2	15 0	2 0	<i>Trace</i>	„	0	0	
28	4	7 5	<i>Trace</i>	0	0	0	0	
29	3	9 0	„	0	0	<i>Trace</i>	<i>Trace</i>	
31	2	12 0	„	<i>Trace</i>	<i>Trace</i>	0	0	
33	4	86 0	4 0	„	„	<i>Trace</i>	<i>Trace</i>	Trace till 6th day
32	6	36 5	2 0	„	„	„	0	
32	7	8 9	3 0	2 0	1 0	„	0	
18	2	7 5	5 0	3 0	1 5	„	<i>Trace</i>	Trace till 9th day
26	1	16 0	2 0	1 0	<i>Trace</i>	1 0	0	
41	12	13 5	1 0	1 0	„	<i>Trace</i>	0	
37	10	4 0	<i>Trace</i>	<i>Trace</i>	„	0	0	

TABLE III

Showing the percentage of opium addicts and the presence of morphine in the urine in a series of 30 treated and 24 non-treated cases after sudden withdrawal of opium

Days after with drawal of opium	TREATED CASES		REMARKS	NOT TREATED CASES		REMARKS
	Number	Per cent		Number	Per cent	
1st day	30	100 0	N B—Urine in all these cases became morphine free after 7th day	24	100 0	N B—Urine in all these cases became morphine free after 10th day
2nd "	25	83 3		22	91 6	
3rd "	18	60 0		21	87 5	
4th "	4	13 3		13	54 1	
5th "	3	10 0		10	41 6	
6th "	None	0		6	25 0	
7th "	1	3 3		4	16 6	
8th "	None	0		3	12 5	
9th "	"	0		2	8 3	
10th "	"	0		1	4 16	

COMPLEMENT-FIXATION IN HYDATID DISEASE SUGGESTIONS

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THE main difficulty in this reaction appears to be the lack of a stable and standard antigen Fairly (quoted by Dew, 1928) advocated three kinds of antigen (i) fluid from hydatid cysts of sheep, (ii) saline extract of scolices and (iii) alcoholic extract of scolices (diluted with normal saline) The first he considered the best For precipitin and skin reactions a phenolized hydatid fluid was recommended McIntosh (1931) is also of the opinion that 'for the diagnosis of hydatid disease the best antigen is the fluid from a hydatid cyst collected aseptically and distributed in sealed tubes' The writers phenolize, select and pool fluids for their complement-fixation technique which is linked to their Wassermann reaction

THE ANTIGEN

From carcasses of freshly slaughtered sheep fluids from a dozen or so cysts are collected by aspiration in separate bottles To each bottle is added on the

spot 0.5 per cent of a mixture of equal parts of ether and trikresol. The bottles are shaken vigorously, brought to the laboratory and left in a refrigerator overnight. Next day the fluids are tested for anticomplementary and hæmolytic activity.

For determining the anticomplementary activity one volume (0.25 c.c.) of each fluid is added in a tube to one volume of a complement dilution containing 1 m.h.d. as determined for the Wassermann reaction done after Method No. 4 of the (British) Medical Research Committee (now Council) (1918), and one volume of saline. The mixture is left for half an hour at room temperature and then incubated for half an hour at 37°C. One volume of standardized (Greval, 1929, Greval, Yesudian and Choudhury, 1930) and sensitized suspension of the red blood cells is then added and the tubes incubated again for half an hour. A fully lysed tube (not necessarily crystal-clear) indicates that the phenolized fluid, from which one volume (0.25 c.c.) will be used for the test, is not anticomplementary. If a fluid is anticomplementary (the tube charged with it is not fully lysed), it is rejected.

For determining the hæmolytic activity three volumes of a selected fluid are added in a tube to one volume of the red cell suspension and incubated. There should be not a trace of lysis (confirmed by leaving the tubes in a refrigerator overnight). The fluid showing hæmolytic activity is rejected. The fluid discolouring the cells without discharging the colour is also rejected.

At least six selected fluids are pooled to yield a standard antigen. This antigen remains stable for at least a year, in a refrigerator. For use the bottle is shaken, the coarse particles allowed to settle and the uniformly opalescent fluid removed.

A positive known serum diluted 1 in 10 with saline containing 0.25 per cent phenol also keeps well, after an initial drop in titre, in a refrigerator. It is used as a positive control in the test of the preserved antigen.

The standardization of the antigenic fluids attempted so far is not final. The writers do not know the probable average limit of the reaction of the positive sera. When the limit is known the fluids will be further standardized by selection after observing their reactions with titrated controls of pooled positive sera.

Phenol added to the fluid does not interfere with the hæmolytic system. Besides preserving the fluid it definitely increases its antigenic power of fixing complement and thus acts as a fortifying agent [*cf.* fortifying action of phenol on Wassermann antigen (Greval, Chandra and Das, 1939)].

In Calcutta a dozen fluids can be obtained sometimes on one day from the municipal slaughter-house.

THE TEST

The work is undertaken on a day when the complement, as determined for the Wassermann reaction, is of optimal reaction and titre (Greval, Chandra and Das, 1940). The serum dilutions used are 1 in 10, 1 in 50, 1 in 100 and 1 in 200. To

each dilution are added one volume (0.25 c.c.) of a complement dilution containing 2 m.h.d. and one volume of antigen. From the 1 in 10 dilution is put up a serum control containing one volume of phenolized saline (0.25 per cent) and one volume of complement (no antigen). The tubes are left at room temperature for half an hour and then incubated at 37°C. One volume of the red cell suspension is now added and the tubes incubated again at 37°C for half an hour. The ensemble at the end of this communication gives this scheme.

The result of each tube is recorded as +, T (trace of lysis), ± (well-marked inhibition of lysis), ² — (not crystal-clear tube) and —. For detection of T the tubes are left in the refrigerator overnight.

Only a + is reported as positive. The report reads 'positive in 1 in 100' or 'positive in 1 in 50' and so on. The serum control of course must be also fully lysed, if not in a 1 in 10 dilution, at least in a dilution considerably lower than the one which gives a + reaction.

A serum dilution of 1 in 5 may be tested if the 1 in 10 dilution gives a T or ± reaction.

A Wassermann reaction is done on the same serum at the same time.

In this technique which is linked to the Wassermann reaction the general plan followed is the same as in complement-fixation for leprosy (Greval, Lowe and Bose, 1939) and kala-azar (Greval, Sen Gupta and Napier, 1939) which are also linked to the Wassermann reaction in this laboratory. For details re variation in the dose of the serum versus variation in the dose of the complement and possibility of paradoxical reaction communication on the latter subjects may be consulted.

The writers' experience of positive reactions is limited. In the experience of the surgeons the hydatid disease is very rare in Calcutta. Cases suspected by physicians have been found to be serologically negative and on rare occasions doubtful (T or ±) during the last ten years. For the keeping property of preserved fluids and the relative proportion of the reagents in the test serum from a surgically positive case was used. It had been positive in a 1 in 100 dilution.

THE *Cysticircus cellulosæ*

The hydatid cyst in the sheep is *Cysticircus granulosus*. The usual hydatid in man is the same. For the detection of the unusual *Cysticircus cellulosæ* in man the fluid from the corresponding hydatid cysts in the pig will be a better reagent. These cysts, however, are not so readily procurable. It is not known to the writers how far a group reaction with the fluid from the hydatid cysts in the sheep succeeds in detecting the unusual *Cysticircus cellulosæ* hydatid cysts in man. Such a reaction is possible.

It is worth noting that infestation in sheep in Calcutta is common, while infestation in man is very rare. This is held to be true of whole of India and also of Egypt and China (Dew, *loc. cit.*)

THE USE OF THE TEST IN DETECTING RETROGRESSION OF THE HYDATID DISEASE

The test done with a standard or even constant antigen and a standardized hæmolytic system, such as should be used in Wassermann reaction, can give measurable and repeatable readings. More dilutions of the serum can be tested. They should indicate whether the hydatid disease is retrogressing as a result of a treatment other than surgery, such as X-ray. This treatment has been given by Tyzzer (1933). Medicinal treatments of systems other than European allopathy and of homœopathy are always on trial in India.

An ensemble of the test

Tubes Reagents	1	2	3	4	5
	Serum control	(T e s t p r o p e r)			
Serum dilution, one volume of —	1 in 10	1 in 10	1 in 50	1 in 100	1 in 200
Complement, one volume containing —	2 m h d	2 m h d	2 m h d	2 m h d	2 m h d
Antigen	<i>Nil</i>	1 vol	1 vol	1 vol	1 vol
Saline	1 vol	<i>Nil</i>	<i>Nil</i>	<i>Nil</i>	<i>Nil</i>
Left at room temperature for half an hour and incubated at 37°C for half an hour					
Sensitized r b c suspension	1 vol	1 vol	1 vol	1 vol	1 vol
Incubated at 37°C for half an hour					

Results read immediately and next day after leaving the rack in the cold overnight

SUMMARY.

1 At least six phenolized, selected and pooled hydatid fluids from cysts of freshly slaughtered sheep yield a reliable and stable antigen for a complement-fixation test. It is kept in a refrigerator.

2 The test is linked to the Wassermann reaction.

3 A group reaction with sera from cases of hydatid cysts of *Cysticercus cellulosæ* is possible. The fluid from the corresponding cysts in pigs is not readily procurable.

4 The complement-fixation test as described can give measurable and repeatable readings which should indicate retrogression of the hydatid disease as a result of non-surgical treatment.

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SEASONAL VARIATIONS IN THE INCIDENCE OF FILARIAL LYMPHANGITIS

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INTRODUCTORY

THE number of patients admitted for filarial lymphangitis at the Filariasis Clinic of the Calcutta School of Tropical Medicine varied from month to month. This was observed to be the case from year to year for the last several years. It is well known that filarial parasites (*Wuchereria bancrofti*) are subject to seasonal influences. The percentage of infection and the intensity of infestation as also the time taken by the embryos to complete their development in the mosquito vary from season to season. It is found that during the monsoon months when the humidity is high (80° to 90°) and the temperature optimum (80°F to 90°F) the heaviest infection and a high degree of infestation occur in the mosquito. During the other seasons infection rate is very low, infestation small and the time taken for the completion of the development much prolonged. The transmission of this parasite is also known to vary from the optimum conditions of temperature and relative humidity. The variation in these factors is held to be responsible for the difference in the intensity of filarial infection in different areas. For example, filarial infection rate in the city of Calcutta is 9 per cent, while the infection rate in the central wards of the towns of Puri and Cuttack is 27 per cent and 34 per cent respectively. This difference in the rate of infection is explained by the following facts. A satisfactory drainage system and good sanitary conditions in Calcutta reduce the number of *Culex* breeding places in the city. During the monsoon months owing to heavy rainfall few permanent breeding places are found but when the winter sets in the breeding places are more numerous, and with the decay in organic matter and other suitable conditions there is an increase in the *Culex* population in the city. Table I shows the monthly normals of mean temperature and humidity for Calcutta for the period 1929-1938. It will be seen from the table that the period

from July to September is the most favourable time for the development of the filarial embryos in the mosquito but want of synchronization during this period with the *Culex* factor keeps the infection rate in Calcutta so low. In the towns of Puri and Cuttack, on the other hand, the filarial infection rates are very much higher on account of a larger *Culex* population in monsoon months. This is due to the fact that these towns afford a larger number of suitable breeding places for the *Culex* on account of defective drainage systems and the presence of a large number of sump-pits for sullage water. The third factor of importance in the variation of filarial infection is human crowding, in other words, the number of carriers of filarial infection in an area.

TABLE I

Month-by-month mean temperature and humidity for Calcutta for the period 1929-1938

Months	Mean maximum, °F	Mean minimum, °F	Relative humidity 8 hours * L T per cent	†Relative humidity 17 hours ‡I S T per cent
January	78.7	56.6	84.3	52.5
February	82.7	61.7	84.0	48.8
March	93.6	71.0	79.5	39.8
April	97.0	77.2	78.2	50.7
May	95.5	79.1	81.3	68.7
June	93.2	79.8	85.6	77.5
July	89.1	79.5	89.3	83.0
August	87.8	79.1	89.7	82.7
September	89.4	79.0	89.4	81.7
October	88.8	75.2	86.9	73.2
November	83.4	65.4	83.4	61.8
December	78.4	57.5	84.6	59.3

* L T = Local time † Averages of 6 years only (1933-1938)

‡ I S T = Indian standard time

THE OBJECT

While it is possible that the variation in the number of admissions for filarial lymphangitis month by month may partly be due to several minor factors, such as rain, festivals, etc. it appears from what has been described above that the variations in the admissions month to month may be largely due to seasonal influences. The object of the paper is to ascertain, therefore, from these figures whether we can observe any significant seasonal variations in the incidence of filarial lymphangitis which repeats itself from year to year, and if so to give an objective measure to the relative influences of different seasons.

MATERIAL FOR STUDY

The relevant data are shown in Tables II, III and IV. Table II shows the numbers of new cases admitted month by month, Table III, the numbers of attendances of these cases and Table IV, the annual totals of both.

TABLE II
Numbers of filaria patients admitted month by month

Months	1929	1930	1931	1932	1933	1934	1935	1936	1937	1938
January	62	64	53	60	57	117	103	105	134	114
February	46	50	50	41	59	70	80	116	73	88
March	72	65	79	66	61	75	91	131	128	168
April	68	67	84	70	52	112	77	119	144	159
May	85	56	54	30	104	125	104	107	137	164
June	58	61	98	47	93	79	80	113	139	127
July	78	47	51	73	104	123	125	161	179	187
August	52	61	86	97	114	121	120	160	147	166
September	58	53	101	89	53	127	108	178	141	163
October	41	68	62	61	97	76	144	74	143	111
November	48	61	47	91	90	81	120	120	124	119
December	45	35	38	46	73	55	77	70	80	101
TOTAL	713	688	803	789	957	1,167	1,250	1,490	1,578	1,664

TABLE III

Numbers of attendances of filaria patients month by month.

Months	1929	1930	1931	1932	1933	1934	1935	1936	1937	1938
January	475	635	545	752	557	526	654	737	1,038	1,165
February	511	462	512	538	579	670	832	980	802	1,201
March	527	462	855	417	593	485	845	993	1,121	1,457
April	427	682	625	757	490	608	816	689	1,220	1,334
May	315	698	780	472	788	1,102	813	1,104	1,065	1,002
June	371	624	860	312	886	746	581	911	1,256	852
July	424	709	637	468	854	836	788	1,260	1,499	1,292
August	519	785	870	696	1,112	906	1,200	1,216	1,517	1,425
September	311	523	930	968	737	936	910	1,371	1,474	195
October	307	68	617	452	647	637	806	776	1,076	693
November	443	61	666	930	874	692	1,185	718	1,287	925
December	530	35	635	558	602	613	786	754	1,039	1,162
Total	5,160	4,744	8,532	7,420	8,731	8,817	10,216	11,509	14,454	12,705

TABLE IV

Annual totals of admissions and attendance of filaria patients

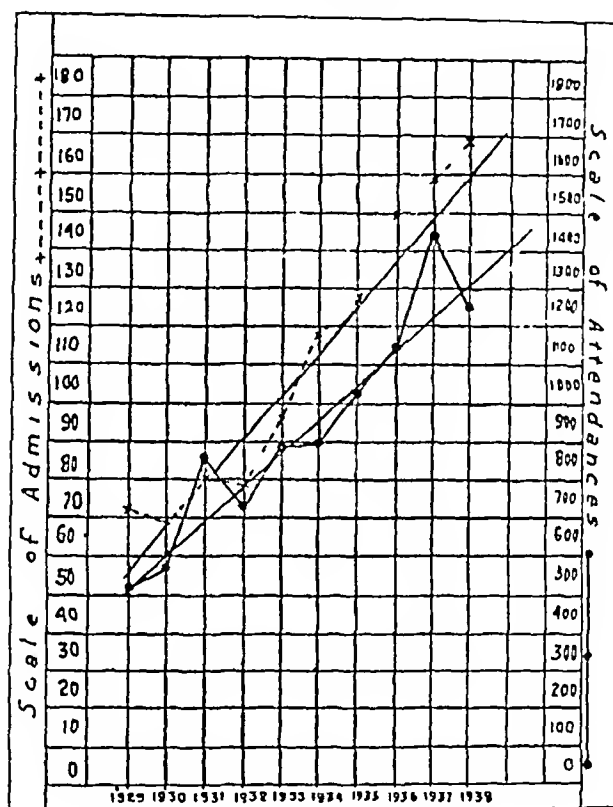
Years	ANNUAL NUMBERS OF ADMISSIONS		ANNUAL NUMBERS OF ATTENDANCES	
	Number of patients registered	Trend numbers	Number of visits	Trend numbers
1929	713	569	5,160	5,177
1930	688	690	5,744	6,100
1931	803	810	8,532	7,022
1932	789	930	7,420	7,945
1933	957	1,051	8,731	8,867
1934	1,167	1,171	8,817	9,790
1935	1,250	1,291	10,216	10,713
1936	1,499	1,412	11,509	11,635
1937	1,578	1,532	14,454	12,558
1938	1,664	1,652	12,705	13,481

It will be seen from Table IV that the numbers of admissions and attendances* have very rapidly increased during the period under review. The tendency of rise is readily perceived from Graph 1. The number of admissions which was only 713 in the year 1929 was doubled during the period of 8 years, reaching 1,499 in the year 1936. In the year 1938 it was 1,664, thus recording an increase of 133 per cent

* The first visit of a patient is an 'admission'. The second and subsequent visits 'attendance'.

on the 1929 number* The number of attendances has shown a similar rise but on a slightly smaller scale The last year is an exception

GRAPH 1
SHOWING THE ANNUAL NUMBERS OF
ADMISSIONS AND ATTENDANCES
OF FILARIAL CASES
1929 TO 1938



THE METHOD.

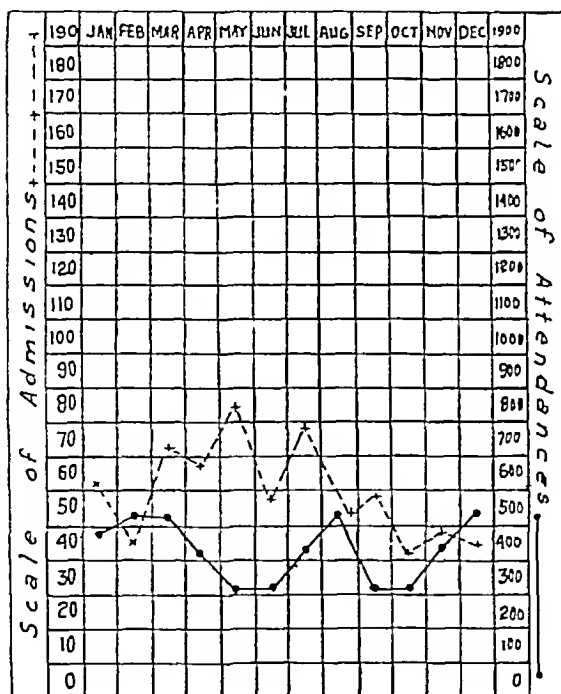
A long-term trend or movement is commonly described by the word 'secular' in statistics. It will be seen from Graph 1 that this secular trend approximately follows the course of a straight line.

Apart from this long-term trend, there will be noticed in Graphs 2 to 11 a type of periodical movement recurring year after year known as the seasonal variation.

*It may be pointed out that this big increase of 133 per cent does not indicate a corresponding increase in the incidence of filariasis in the area served by the Clinic. Indeed, an examination of all the admissions in the Carmichael Hospital for Tropical Diseases, described in the Annual Reports of the Calcutta School of Tropical Medicine, shows that the incidence of filarial infection is fairly constant. The increase may be ascribed to the growing knowledge of the existence of a centre for the treatment of filariasis.

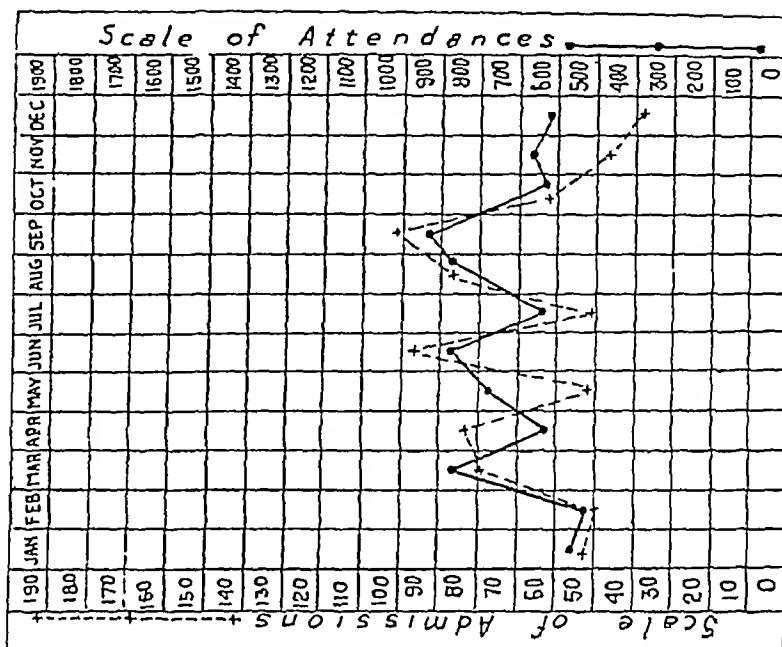
It will be seen, for example, that the numbers of admissions and attendances are more in the monsoon than in the winter months, that their numbers in the month of February are smaller than in the month of January owing probably to the smaller number of working days in that month and so on. The different months will be clearly seen to exert their own typical influences on the numbers of admissions and attendances in virtue of the reasons described in the introductory section, their conventional length and other minor factors.

GRAPH 2
SHOWING THE NUMBERS OF ADMIS-
SIONS AND ATTENDANCES
MONTH BY MONTH
1929

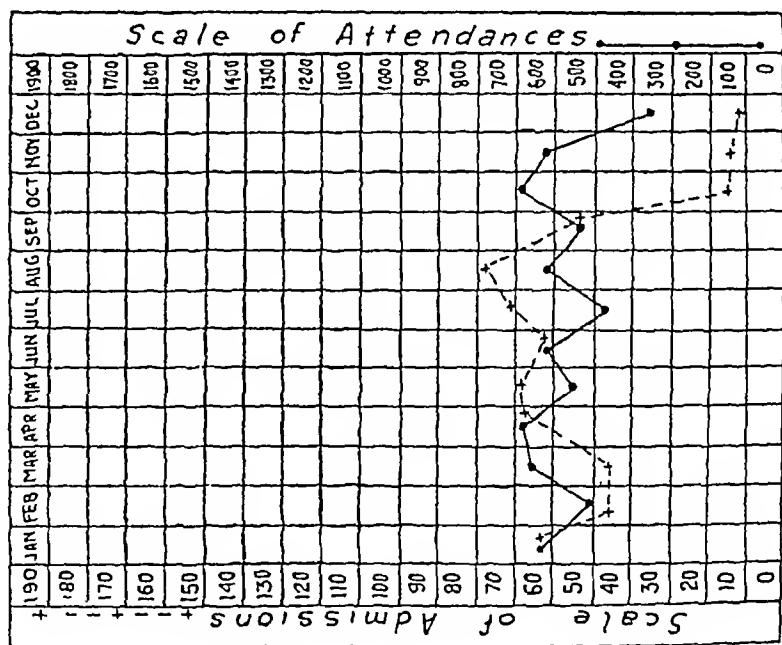


Besides the secular and the seasonal variations, a time-series is usually affected by what is known as the cyclical and random variations. The cyclical variation gives a sort of wave-like appearance to the series and consequently resembles the periodic variation but does not repeat itself after fixed periods with the same amplitude as in the case of seasonal variations. The period of cyclical variation is also much longer than a year. The random variation, as the term suggests, does not obey any law, it is brought about by ordinarily unknown and accidental minor cause. If Graphs 2 to 11 are read together in a series it will be seen that there is

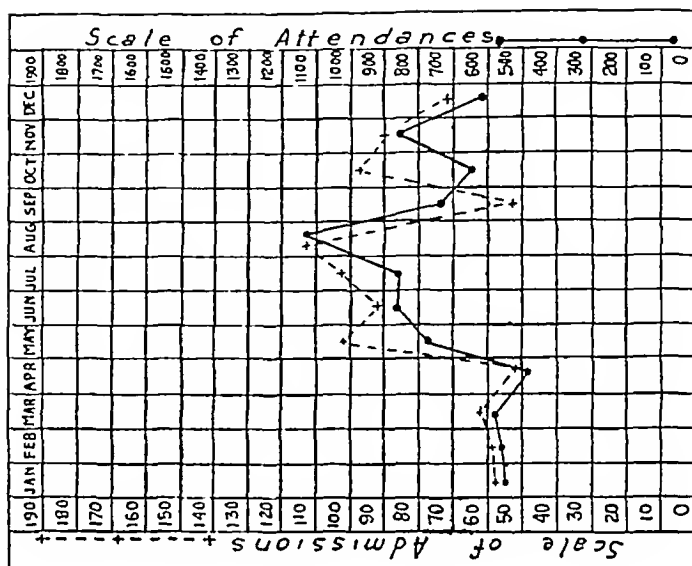
GRAPH 4
SHOWING THE NUMBERS OF ADMIS-
SIONS AND ATTENDANCES
MONTH BY MONTH
1931



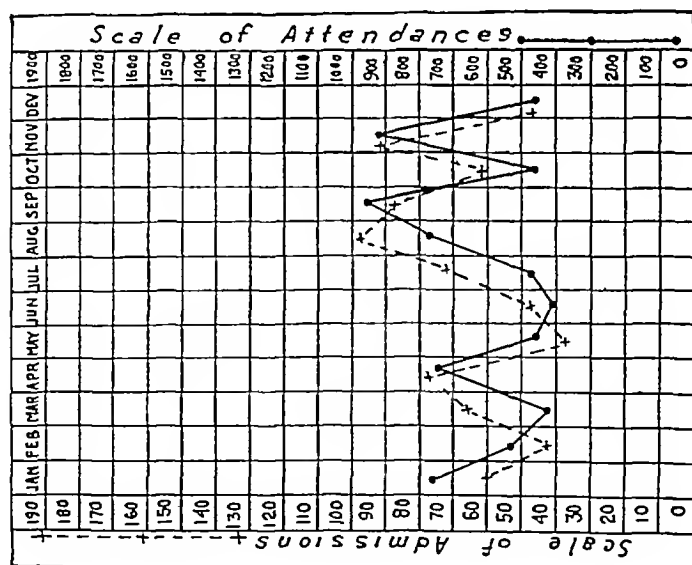
GRAPH 3
SHOWING THE NUMBERS OF ADMIS-
SIONS AND ATTENDANCES
MONTH BY MONTH
1930



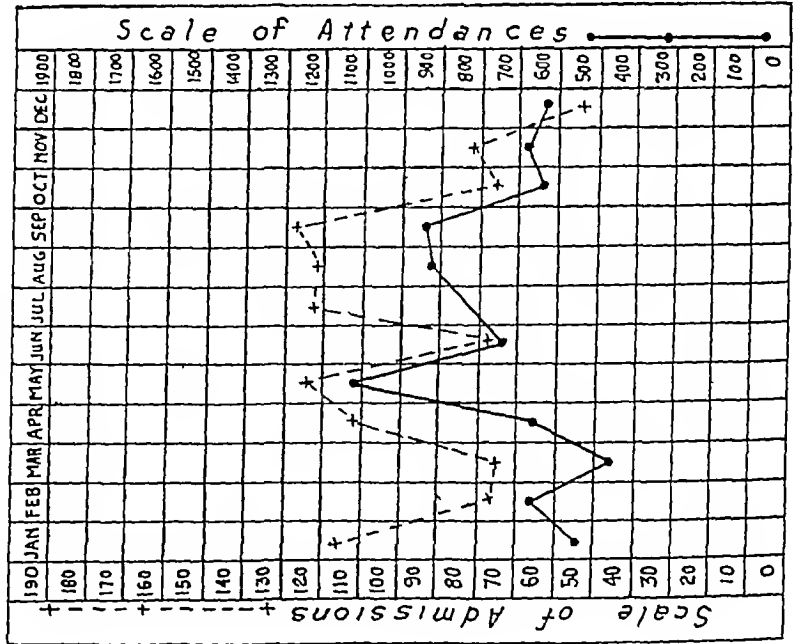
GRAPH 6
SHOWING THE NUMBERS OF ADMIS-
SIONS AND ATTENDANCES
MONTH BY MONTH
1933



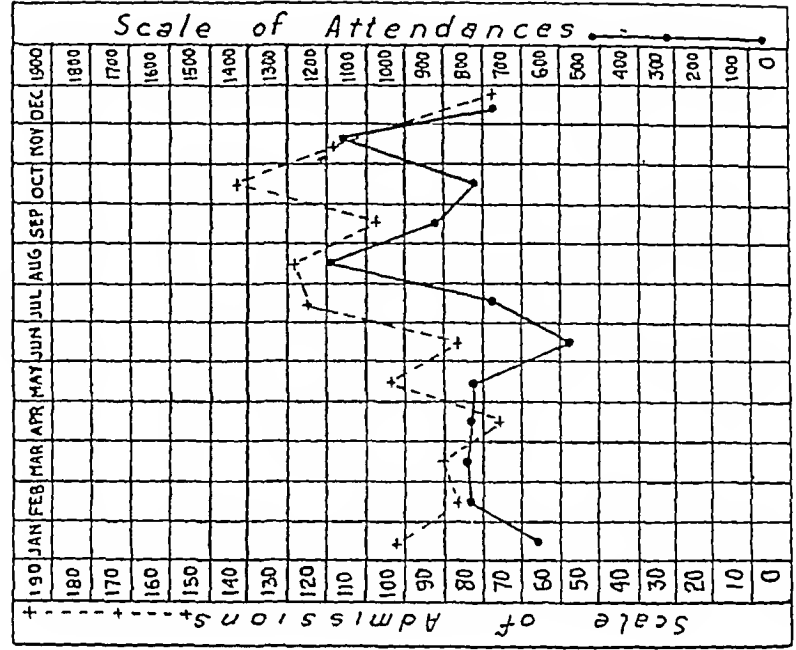
GRAPH 5
SHOWING THE NUMBERS OF ADMIS-
SIONS AND ATTENDANCES
MONTH BY MONTH
1932



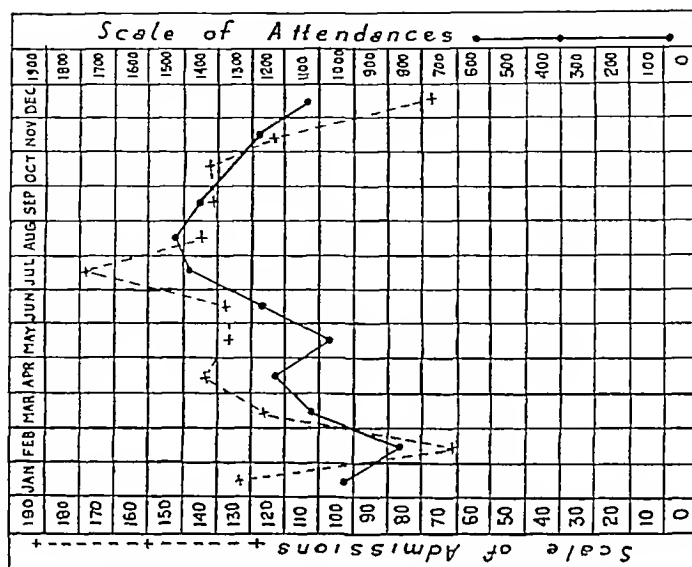
GRAPH 7
SHOWING THE NUMBERS OF ADMIS-
SIONS AND ATTENDANCES
MONTH BY MONTH
1934



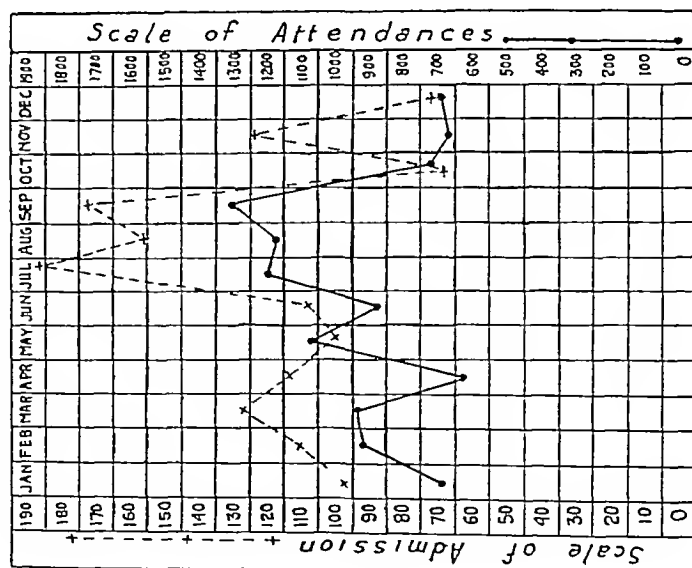
GRAPH 8
SHOWING THE NUMBERS OF ADMIS-
SIONS AND ATTENDANCES
MONTH BY MONTH
1935



GRAPH 10
SHOWING THE NUMBERS OF ADMIS-
SIONS AND ATTENDANCES
MONTH BY MONTH
1937



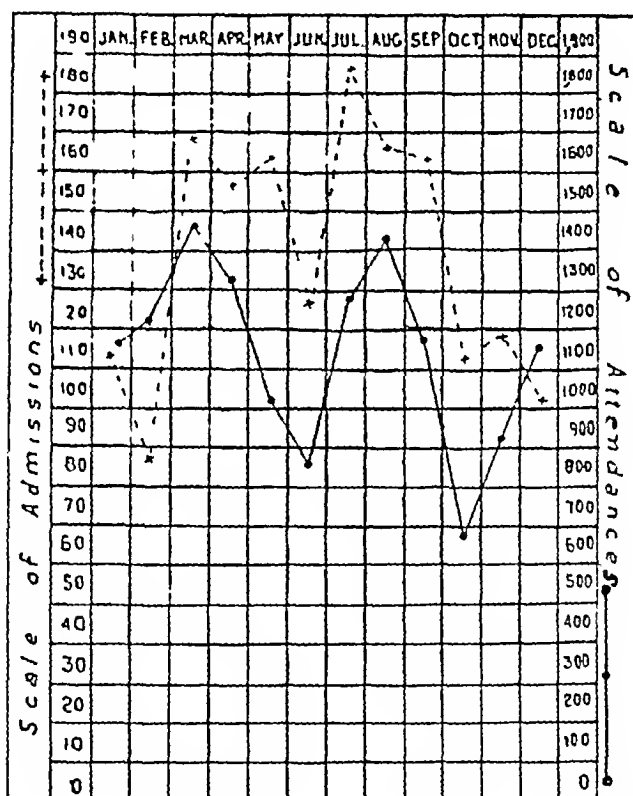
GRAPH 9
SHOWING THE NUMBERS OF ADMIS-
SIONS AND ATTENDANCES
MONTH BY MONTH
1936



no marked cyclical variation but that there are 'random' minor fluctuations which cannot be accounted for by either long-term or seasonal variations. Even if there be a cyclical variation present in a minute degree, the period under review is too small to ascertain its definite existence with specifications of period and amplitude.

GRAPH 11

SHOWING THE NUMBERS OF ADMIS-
SIONS AND ATTENDANCES
MONTH BY MONTH
1938



It will be clear from what has been said above that in order to obtain measures of seasonal variation it is necessary to eliminate the influences of the other variations affecting the series. This is done in the way described below —

It has been shown that the secular trends of both admissions and attendances follow approximately the course of straight lines. The equations to the straight lines which give the closest representation of these trends are found to be—

- I $Y = 1110.8 + 120.4 x$ for series of admissions
 II $Y = 9328.8 + 985.2 x$ for series of attendances

where Y represents the calculated trend value of the numbers of admissions and attendances and x is an integral value in years measured from the mid-year of the year 1933 as origin. The calculated trend values corresponding to these two equations are shown by the side of the actual annual numbers of admissions and attendances in Table IV, and are also shown in Graph 1. It will be seen from a comparison of the actual annual numbers of admissions and attendances with the trend numbers that the correspondence between the two is very close.

A second or a higher degree curve might give a closer fit, but the object of fitting the curve in the present study is not one of forecasting, which indeed it cannot be with time as the only independent variable, but is simply one of adequately representing the long-term trend in the actual time series. If a curve of too high an order is fitted deviations from the trend would contain less of cyclical variations than is actually present. On the other hand, the deviations from an inadequate trend are likely to be highly correlated owing to the presence of a portion of the long-term variation. Graph 1, as well as Table V giving the values of the respective residual variance (variation of the fitted values about the actuals) obtained by fitting a straight line and a parabola indicate clearly the adequacy of the straight-line trends.

TABLE V

	SQUARE ROOT OF RESIDUAL VARIANCE		SQUARE ROOT OF RESIDUAL VARIANCE AS PERCENTAGE OF THE AVERAGE	
	Series I	Series II	Series I	Series II
Straight line	87.4	1,007.5	7.9	10.8
Parabola	91.4	1,072.8	8.2	11.5

Note.—Series I represents the numbers of admission and series II the numbers of attendance.

From the annual trend values the monthly ones are obtained on the assumption that the annual number is centred at the middle of the year, viz. the 1st July, and the monthly is centred at the middle of the month, viz. the 15th. Thus, for calculating the monthly trend number of patients admitted in January 1931, the following procedure is followed—

The trend numbers for the two years 1930 and 1931 are 569.1 and 689.5 respectively. Assuming these to be centred as on the 1st July, 1930 and 1931 respectively, the monthly increment in the annual numbers works out as $\frac{1}{12}$ (120.37) and the monthly increment in the monthly number as $\frac{1}{144}$ (120.37) or 0.836. The July value for trend of 1930 centred at the 1st is $\frac{1}{12}$ (569.1) or

47 427 The July number centred at the 15th is $47\,427 + \frac{1}{2} (0\,836)$ or 47 845
 January number centred at the 15th is $47\,845 + 6 \times (0\,836)$ or 52 861 The
 February value is $47\,84 + 7 \times (0\,84)$ or 53 697 and so on

The easiest way of eliminating the long-term variations from the two series is to take the percentage ratios of the actual monthly numbers with the trend numbers. Space does not permit their tabulation in the paper. Their examination shows that the series of percentage ratios are independent of the secular movements.

It remains to free the two series of percentage ratios from the other two movements, viz the cyclical and random movements. There is, however, no need to follow any elaborate procedure for this purpose, for by virtue of their nature the sum totals for each month of the cyclical and the random changes rarely differ from zero.

The frequency distributions of the percentage values for each month give the whole of the relevant information on the relative influences of different months on the numbers of admissions or attendances in those months. These have not been shown in the paper. When these are tabulated they show that the different distributions in either table are centred not round the same but widely different values. The best estimates of these central values are commonly spoken of in 'Statistics' as the measures of seasonal influences or indices. These estimates are shown in Table VI —

TABLE VI

Months	Series I	Series II
January	96	95
February	78	93
March	105	100
April	111	106
May	105	102
June	99	97
July	122	108
August	119	127
September	114	111
October	89	76
November	98	100
December	63	82
AVERAGE	100	100

CONCLUSIONS

It will be seen from the series of seasonal indices for numbers of admissions that the monsoon months (July to September) are the most favourable for the incidence of the filarial infection. This was expected from the considerations given in the introduction. The monthly incidence during this period is seen to be about 20 per cent higher than the average incidence during the year and about 40 per cent higher than in the winter period from October to February. The period from March to June shows a slightly higher incidence than in the average month but is not statistically found to be significant.

SUMMARY

Statistical analysis of the variation in monthly admissions of patients, new and old, at the Filariasis Clinic of the Calcutta School of Tropical Medicine, during the period from 1929-1938, is carried out with the object of defining precisely the nature of the periodical movement recurring year after year in the incidence of filarial lymphangitis. The analysis showed that the monthly incidence during the monsoon period from July to September when the humidity is high and the temperature optimum is 40 per cent higher than in the winter period from October to February. The period from March to June showed an incidence slightly higher than the average giving an appearance of a prelude to the monsoon period of the highest incidence.

STUDY ON THE NORMAL POLYNUCLEAR (ARNETH) COUNT AT HYDERABAD-DECCAN -

BY

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AND

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[Received for publication, September 30, 1940]

ALTHOUGH the diagnostic value of the polynuclear count in infective state is well established, little attempt has been made in India to find the normal values Banerjee (1924), using Minor and Ringer's modification of Arneth's classification, examined 100 healthy Bengali students and found a shift to the left. He, however, gave no detailed account of his work.

That the values established for colder climates may not be suitable to tropical countries is brought out by the work of Kennedy and Mackay (1936) who investigated the polynuclear count in 271 British airmen in Iraq and the neighbouring countries. They found marked left deviation of the count. The neutrophils were relatively reduced in number, with the corresponding increase in the monocytes. These changes were attributed by them to be due to the climate of the country.

MATERIAL AND METHOD

The subjects selected were 120 men out of whom 20 were medical students. The rest were selected from the general population of Hyderabad, their ages varying from 15 to 45 years. Only those subjects were selected who gave a history of good health and had not suffered from any minor illness such as common cold for at least 4 weeks and from any major illness such as an attack of malaria or dysentery for at least 6 months prior to examination. Teeth, gums and tonsils, being the most common foci of infection, were examined with particular care. Under such criterion, it was possible to select only 20 students out of 100 examined for the purpose. The rest had to be selected from the general population of the town.

The blood was taken from finger-prick and the film fixed by immersion in a saturated solution of mercuric chloride for 15 minutes and then stained for 24 hours with the iron-haematoxylin method. We found this technique quite satisfactory for the polynuclear count, the nuclei were deeply stained and the filaments between the lobes were easily visible. Cooke and Ponder's (1927) criterion of nuclear division was observed, viz 'if there was any band of nuclear material except a fine chromatin filament connecting the different parts of a nucleus, that nucleus could not, for the purposes of the count, be said to be divided'. A total leucocyte count was also made in each case, a Neubauer double-counting chamber being employed and an average of four counts being taken. Both the pipette and the chamber used were those certified by the American Bureau of Standards. The planeness of the surface of the haemocytometer cover-glass was tested with Hausser's interferometer. The blood for these counts was taken without any regard to the time of the day and without any particular relation to the time of the meal. A differential count was also made at the same time, 200 cells being counted, the film for the purpose having been stained with Leishman's stain.

RESULTS

The result of the differential count is given in Table I. Very few 'abnormal' cells were seen in these subjects. Myelocyte was seen only once. Three of the subjects showed large lymphocytes with deeply staining cytoplasm resembling Turk cells, one each in a count of two hundred cells. In two cases lymphocytes with double nuclei were seen. In subject No 36 a large neutrophil was seen with hypersegmented nucleus resembling the macropolycyte of Cooke and Ponder (*loc cit*).

TABLE I

Differential counts	Mean	Standard deviation	Maximum	Minimum
Neutrophils	55.0	10.48	75.5	32.0
Lymphocytes	32.0	9.08	53.0	15.0
Monocytes	5.5	3.63	18.0	1.0
Eosinophils	7.0	4.52	23.0	1.0
Basophils	0.5	0.52	3.0	0.0

The average leucocyte count for the 120 subjects works out as 7,224 with the standard deviation of 1,228. The lowest count was 4,000 in subject No 53 and the highest count was 11,000 in subject No 99. The details of the leucocyte count are classified in Table II, the cumulative frequency curve or ogive of which is shown in Graph 1.

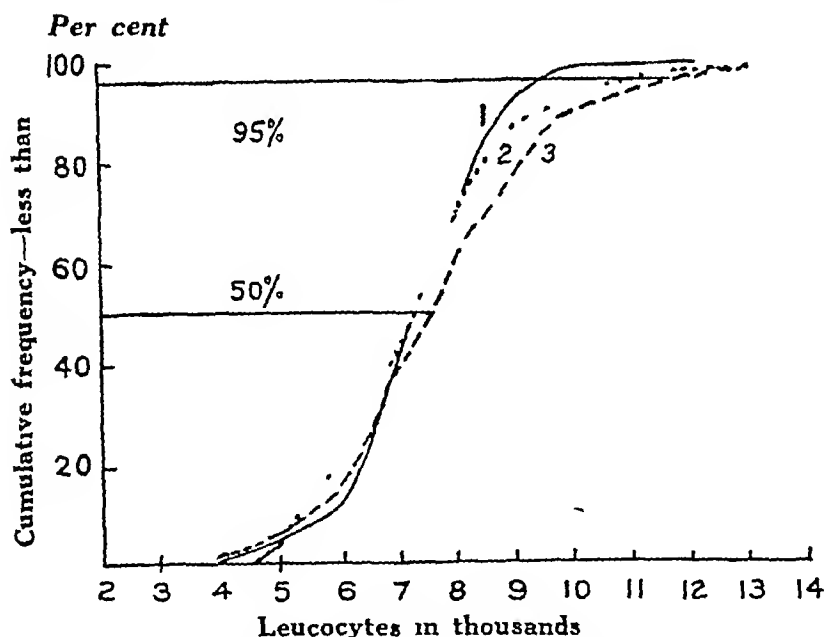
TABLE II

Classified according to the number of cells per
c mm of blood (cumulated upwards)

Number of cells per c mm of blood <i>Less than</i>	Number of counts	Per cent
5 000	6	5.0
6 000	15	12.5
7,000	51	42.5
8,000	87	72.5
9 000	111	92.5
10,000	119	99.1
11 000	119	99.1
12,000	120	100.0

For the sake of comparison other cumulative frequency curves based on the work of Galambos (1912, quoted by Garrey and Ray Bryan, 1935) and Osgood

GRAPH 1



Cumulative frequency distribution curves of leucocyte counts of adults

- (1) Rahman and Zaidi—
 (2) Osgood (1934)— random counts
 (3) Galambos (1912)— random counts
 throughout the day

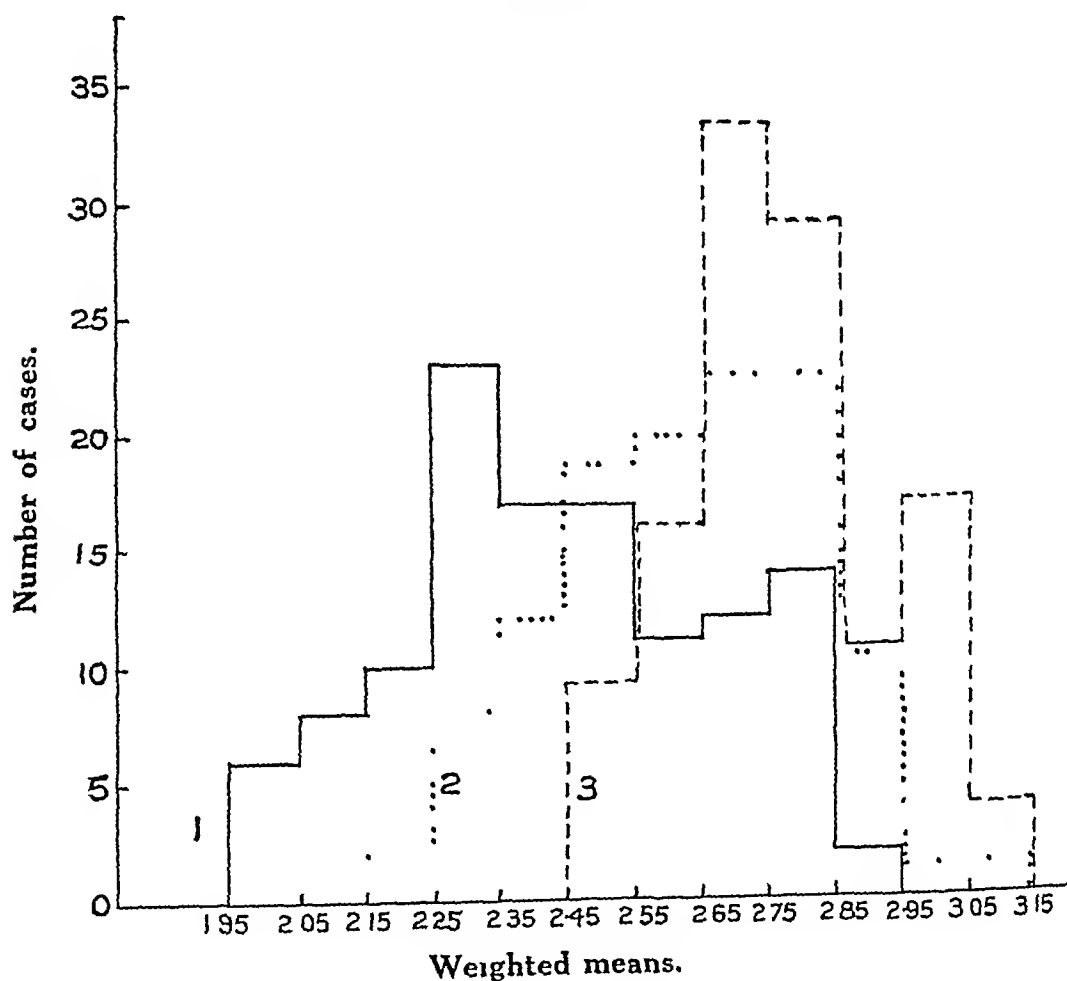
(1934), in which samples of blood were taken at random during the day, are superimposed. Close resemblance is seen between these curves.

The mean of the polynuclear count works out as 2 432, the standard deviation being 0 234. The nearest count corresponding to this mean in the series was —

I	II	III	IV	V
12 8	39 0	39 0	8 2	1 0 — — — 2 456

The result of the polynuclear count is represented in the shape of a frequency distribution polygon (Graph 2). For the sake of comparison the polygons based on the results obtained by Kennedy (1933) and by Cooke and Ponder (*loc cit*) are superimposed, converted to the same scale.

GRAPH 2.



Frequency polygons based on the results obtained by —

(1) Rahman and Zaidi —————

(2) Cooke and Ponder (1927) — .

(3) Kennedy (1933) — - - - -

DISCUSSION

It is seen from Graph 2 that the results obtained by us differ greatly from those obtained in the European subjects by Cooke and Ponder, our results show a marked deviation to the left and the range is larger so that the polygon is diminished in height and increased in breadth. The latter factor is of importance from clinical point of view as the chances of an abnormal count falling within the normal range are increased.

The polygon based on the results obtained by Kennedy shows significant differences as compared with that based on the results of Cooke and Ponder (Graph 2). Kennedy's subjects were medical students at Edinburgh. The mean of the counts on 90 subjects studied by him was 2 628 corresponding to a count of—

I	II	III	IV	V
13	30	43	10	4

Whereas the average mean of the 90 subjects studied by Cooke and Ponder was 2 74 corresponding to a count of—

I	II	III	IV	V
12	25	44	15	4

Kennedy's results thus show not only a significant deviation to the left but, as is evident from the polygon in Graph 2, the range in which the counts fall is also increased. This difference has been attributed by him to be due to the relative laxity of the health standard of his subjects as compared with that of Cooke and Ponder.

The results obtained by us show even more marked deviation than those of Kennedy. We do not attribute this difference to be due to the laxity of the health standard of our subjects as reasonable care was taken in selecting healthy subjects. In subjects who showed a high eosinophil count, stools were examined to exclude parasitic infections. The teeth and lungs were, however, not X-rayed and this might have admitted a few cases with hidden foci. Even admitting such a possibility, the deviation in the results is so marked that it cannot be due to this factor alone. That the counts show a stable state is evidenced by the fact that no double maxima occur in the count and that the total leucocyte count falls within the normal range.

There is every reason to believe that the tropical climate by itself would bring about and maintain a left-handed deviation of the polynuclear count. The work of Kennedy and Mackay in this connection has been mentioned above. They found the weighted mean of the polynuclear count for British airmen in Hinaidi to be 1 935. They found also increased number of 'abnormal' cells in the blood of their subjects, affording an evidence of bone-marrow stimulation. They also found a relatively large percentage of monocytes and correspondingly low percentage of neutrophils. These effects were attributed by them to be due to the climatic causes.

In the results obtained by us the monocyte count is not any more than what is considered normal according to the European standards. The increase is noticed in the lymphocyte and the eosinophil counts with the corresponding decrease in the neutrophils. The total leucocyte count, as indicated by Graph 1, is not much different from that which has been observed in the European subjects. That the tropical climate may give rise to increase in lymphocytes has been stated by Russell and Russell (1928, quoted by Kennedy and Mackay, *loc cit*) on the basis of observations on a large number of soldiers in a temperate climate, and after their transfer to the tropics. It is interesting to note that Napier and Das Gupta (1935) found the eosinophil count in 50 adult male Indians working in Calcutta to average 6.91 ± 5.19 per cent, figures which are almost identical with ours.

It is therefore suggested that the deviation to the left, as compared to European standards, of the polynuclear count in our subjects is, in all probability, due to the climatic factor. If we accept the view that the neutrophils with greater number of lobes are more mature than those with fewer lobes, then, as the total leucocyte count is not altered, it would mean that the span of life of the neutrophils in these subjects is less and their rate of production correspondingly greater than that occurring in the European subjects. These two factors combined would maintain a larger population of young cells in the circulation without altering the total neutrophil count.

SUMMARY

1 A study of polynuclear count was made in 120 healthy male subjects at Hyderabad-Deccan. Their ages ranged from 15 to 45 years. Total leucocyte counts and differential counts were also made.

2 The average weighted mean of the polynuclear counts was found to be 2.432 with the standard deviation of 0.234. This average is much less than that established by Cooke and Ponder for European subjects. The cause for this low figure is discussed. The total leucocyte count was not found to differ appreciably from the results obtained in colder climates. The lymphocyte and the eosinophil counts were found to be higher and the neutrophil count correspondingly lower than those given for the European subjects.

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ON ISOHÆMAGGLUTINATION NOMENCLATURE,
TITRATION OF ISOHÆMAGGLUTININS, NEED
FOR REVISION OF TECHNIQUE OF
GROUPING BLOOD, ETC

BY

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[Received for publication, September 30, 1940]

DURING the last 4 or 5 years blood groups in India have been steadily gaining in importance. Provincial Red Cross Societies are organizing depots for blood transfusion service. In Calcutta where such a service has been run from this laboratory for the last 16 years the demand for donors of blood increased recently and threatened to exceed the supply. In the following pages will be dealt with (i) the nomenclature, (ii) the titration of the isohæmagglutinins, (iii) the quantitative relation between the isohæmagglutinins and the isohæmagglutininogen as they exist in the body and for a reaction, (iv) the need for a revision of the technique of grouping blood and (v) a revised technique.

I THE NOMENCLATURE

Plate VIII shows the serological constitution of blood groups and also the old equivalents of the new terms. In the interest of economy and ease in speech the terms isohæmagglutininogen and isohæmagglutinin have been replaced by 'isogen' and 'isonin' respectively. It is suggested that they should be so known. For the same reason hæmagglutininogens (not iso) M and N have been called hæmogens M and N. The term 'factor' is unnecessary.

With respect to the cells and subjects it is suggested that the qualifying letter denoting the isogen should invariably follow the noun, e.g. cells A, subject A, donor A, recipient A

With respect to the serum it is suggested that in conformity with the cells it should be named after its isonin content denoted by small letters corresponding to the capital letters. The small letter should also follow the noun, e.g. serum a (from subject B), serum b (from subject A), serum ab (from subject O) and serum o (small letter, from subject AB). α and β are not necessary.

The serum a absorbed with cells A_2 is serum a_1 . It is used in differentiating between A_1 and A_2 . Sera a_2 and a_3 (occurring abnormally) can be so designated and discussed.

Whether a capital or small letter is meant need not be mentioned. 'Cell A' cannot be 'cell a', nor can 'serum a' be 'serum A'. The position of the noun before the letter indicates whether a capital or a small letter is meant.

The blood is *grouped* when O, A and B are determined and *typed* when M and N are determined.

The fluids used in determining M and N are antifuuids.

In a defective group an isonin which could exist compatibly with life is absent, e.g. Oa (instead of Oab).

Other terms suggested in this communication are 'slow A's', 'centrifugal' agglutination and 'centripetal' agglutination. Explanations concerning them will be found in the narrative in which they occur.

Unfortunately for the denotations of letters some early workers (Coca, 1918, Feinblatt, 1926) used the small letter for the isogen and the capital letter for the isonin. This should be forgotten.

II THE TITRE OF THE ISOHÆMAGGLUTININS (ISONINS)

1 *Definition*—In this work the term titre denotes the highest dilution of serum, which, when added to an equal volume of a 2 per cent suspension of the appropriate red blood cells, agglutinates the latter. It is the *initial* dilution not the *ultimate* dilution reached in the total fluid. This definition is in accordance with the serological usage followed in estimating the potency of the hæmolytic amboceptor and the complement, in complement-fixation.

In bacteriological usage the titre of an agglutinating serum is given by the *ultimate* highest dilution. This practice is also followed by some workers on blood groups.

The time allowed for the agglutination is 15 minutes at room temperature, although a final reading is also taken after 30 minutes. While weak reactions are sometimes found to have become intensified at the final reading, no new change occurs in the suspension after 15 minutes.

2 *Apparatus required*—The details of the apparatus have been given in a previous communication (Greval, Chandra and Woodhead, 1939). The special

features are (i) calibrated capillary finger pipettes delivering 50 drops to 1 c c , (ii) moist-chambers made with medium-sized Petri-dishes and small flat-bottomed watch-glasses in which small folded filter-papers soaked in water are placed and (iii) glass-rods 4 to 5 inches long with rounded ends to stir and spread serum and cells. A photograph gives the special features and the macroscopic appearance of the isohæmagglutination.

3 *Test suspensions*—Two per cent suspensions of r b c A and r b c B are required. Their preparation has also been given in the previous communication. Each suspension is tested for sensitiveness and specificity against three appropriate and non-appropriate sera. For the purpose of testing and titrating unknown sera only the r b c of known healthy subjects, such as donors of blood, are used.

4 *Technique of titration*—For sera ab. This technique was evolved for selecting sera ab of a high and equal titre for absorption test, in determining group from a bloodstain, in medico-legal work.

The clear and inactivated sera from cases not suffering from febrile states, wasting diseases or anæmia, left over from the previous day's Wassermann reaction, are selected. They are contained in small phials suitably labelled. Opposite a batch of four phials are stood four small tubes, $3" \times \frac{3}{8}"$, such as are usually used in Wassermann reaction, suitably labelled to correspond to the phials. A calibrated capillary finger pipette (hereafter called capillary pipette) is dropped into each phial and also into each tube.

Into the tubes is delivered 1 c c of saline. From the corresponding phials is added one drop of serum and recorded on the tube by one vertical line. The serum and the saline are mixed by shaking and the mixture run up and down the capillary pipette three times. The dilution of the serum is 1 in 51 or 1 in 50 (approx.)

A slide is now prepared to test the dilution. The obverse is divided into right and left (operator's) halves by a central line drawn by a glass-marking pencil, on the reverse, along the lower margin, are inscribed the number of the slide (to correspond to the number of the tube) and one vertical line (to correspond to the drop of serum added to the tube), and one drop of the serum dilution is deposited on each half of the slide. Four slides, from four tubes, are so prepared and left in the moist-chamber.

With a calibrated capillary test pipette one drop of suspension of r b c A is dropped on each left hand drop of the serum dilution. With another calibrated capillary test pipette one drop of suspension of r b c B is dropped on each right hand drop of the serum dilution. The suspensions and the serum dilutions are mixed and spread over an area about $\frac{3}{8}"$ in diameter, by the rounded end of a rod moving clockwise and then counter-clockwise, the same number of times for the two collections of fluid on the two halves of the same slide, one end mixing and spreading one collection. On the lid of the Petri-dish is written 'For titre of ab, 10-30 (or a later hour)'.

Results are read (i) in 2 minutes, (ii) after 5 minutes, (iii) after 15 minutes and (iv) after 30 minutes. The slides are kept in the moist-chamber all the time.

The drops are set in motion, before taking the reading, by tilting and untilting the dish with a clockwise and counter-clockwise rotatory motion. Incipient agglutination is read with the aid of a hand lens. A mere sedimentation of the r b c is at once differentiated from the agglutination by the cells arising from the slide, producing the appearance of a thin cloud dispersing before a breeze and forming patterns before going into a homogeneous suspension again. A quick, jerky and side-to-side movement of the dish helps in the re-forming of the suspension. The preparations in the moist-chamber resist drying for 24 hours, the r b c in most of them, however, are found lysed next day. No necessity is ever felt for removing a slide from the chamber for manipulation and inspection. Herein lies the superiority of the macroscopic technique over the microscopic technique. Manipulations and observations can never be made so easily and speedily under a microscope.

If agglutination occurs in 2 minutes, the 1 in 50 dilution is further diluted to 1 in 100 by adding 1 c c of saline to the same tube. The new dilution is tested as before.

The results read after 5 minutes are recorded but not considered final. Two minutes' observation is not needed for any dilution other than the first (1 in 50).

The results read after 15 minutes will be final for most sera. No new change is initiated after 15 minutes. Positive reactions may become more pronounced, doubtful reactions may become positive but negative reactions remain negative. A confirmation of results after 30 minutes, however, is recommended. All the results tabulated in the specimen page (*see* page 252) were confirmed after 30 minutes.

If agglutination does not occur in a 1 in 50 dilution, one more drop of serum is added to the dilution and recorded on the tube by another vertical line. The balance of 1 in 50 dilution consisted of 49 drops. For all practical purposes the first drop of serum added to it was still in it. Another drop added means 2 drops of the serum in a total of 50 drops or a 1 in 25 dilution. The dilution is tested as before.

If no agglutination occurs in a 1 in 25 dilution, one more drop of serum, the third drop, is added to the dilution in the tube, which measures 48 drops. For all practical purposes the 2 drops of serum added previously are still in it. The dilution now is 3 in 49 or 1 in 16 (approx). This dilution is tested as before.

If no agglutination occurs in a 1 in 16 dilution, one more drop of serum, the fourth drop, is added to the dilution which measures 47 drops. For all practical purposes the three previously added drops are still in it. The dilution now is 4 in 48 or 1 in 12.

In the same way further addition of drops are made and stronger dilutions obtained. The outgoing drops, for testing, are looked upon as drops of saline only, the small quantity of serum in them being ignored. The dilutions corresponding to —

1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 drops are 1 in —
50, 25, 16, 12, 9, 8, 7, 6, 5, 4, 4 and 3 respectively

The dilutions are really slightly weaker than is shown.

The advantage of this method of titration is that although 13 dilutions (including the 1 in 100 dilution, not shown in the parallel lines of figures) are tested from each serum only one tube is needed. Four sera can be tested side by side in one Petri-dish. Limitation of space and the corresponding concentrations in observation result.

This rather long series has recently been contracted to 1 in 50, 1 in 25, 1 in 16, 1 in 12, 1 in 9, 1 in 6 and 1 in 3 as is explained in Table I.

For sera a and sera b

The same procedure is followed in dilution. One drop is used in testing with the appropriate r b c and one drop is rejected. The slides are divided on the obverse and marked on the reverse to receive *two dilutions* of the same serum.

5 *Results of titration of 106 sera*—In Table II are given the titres of 37 sera ab, 38 sera a and 31 sera b. The table was completed before the series of dilutions was contracted.

It will be observed that (i) sera ab have higher titres than sera a and sera b, (ii) sera a have a higher titre than sera b, (iii) majority of the sera have a titre between 1 in 25 and 1 in 6, (iv) sharp reactions in one dilution only and spread out reactions in three dilutions are in the minority, the majority of the sera giving reactions spread over two dilutions.

It must be recorded that exclusion of febrile states, wasting disease and anaemia was not as rigidly effected in this series of titrations as is done in selecting sera for absorption.

6 *Mixing of sera a and sera b*—The titre of a mixture of a serum a and serum b, both of known titre, is not usually given by the calculated figure. This probably is due to the presence of varying quantities of the isogens in the plasma. Subjects' isogens are not confined to the r b c or even other cells of the body.

7 *The dangerous 'universal donors'*—The isonins of subjects O 'universal donors' are on the whole stronger than those of subjects A and subjects B. One of the former with a particularly high titre is capable of killing recipient of any other group. Instead of hæmagglutination a hæmolysis may occur and usually does occur, at once or after a delay.

Strange though it may seem opinion on the danger of using these donors is not undivided. According to Keynes (1920) 'theoretically this is an objection to the indiscriminate use of group IV for transfusion, but as no ill effects result such agglutination presumably does not occur in the body'. De Bakey and Honold (1938) have more recently drawn attention to Hesse's statement that 'the transfusion of blood from a universal donor with a titre of 1/16 causes no symptoms but in cases with higher titre signs of hæmolysis are likely to occur'. 'Interesting clinical and experimental observations analysed by E. P. Gesse in a review of twenty-two papers emanating from the First Surgical Clinic and the Research Institute for Blood Transfusion in Leningrad (E. P. Gesse, chief)' abstracted in an Editorial of the *Journal of the American Medical Association* (1936) are traceable

to the same worker, Hesse Wiener (1939) in the second edition of his book also refers to Hesse's observations in a general way

Between the two extremes lie the observations of Townsend, Isabelle and Coca (1936) which make the accidents depend on temperature

The present writers are inclined to believe that accidents of indiscriminate transfusion with blood O have escaped detection. The expectation of life of the recipient who must be given a transfusion as soon as possible, without a determination of group and direct matching of bloods, is not good. It may not be easy to decide whether he has died of the transfusion or in spite of it. The symptoms caused by intravascular agglutinations will not be as apparent as those caused by hæmolysis. In the early days of blood transfusion many recipients were supposed to have benefited after indiscriminate transfusion with bloods of all groups

The writers have definite knowledge of (1) certain donors O who, several years ago, caused severe rigors and collapse, each twice, when used as universal donors and (2) of a hospital which ignoring advice used a donor O as a universal donor and has since refused to use even properly tested and recommended 'safe' universal donors. The extraordinarily quick lytic effect *in vitro* of some fresh sera is well known. They lyse the appropriate red blood cells on a slide before the latter have had time to agglutinate

The original publication of Hesse giving the techniques of titration is not available. Long-distance correspondence has failed to supplement what has been published concerning it. The concentration of the r b c suspensions used in the titration of the isonins is not known, nor is it known whether the term titre indicates the initial dilution or the ultimate dilution. The writers err on the right side and call 'universal donors of the first choice' only those donors O the titre of whose serum is *below* 1 in 16. By all calculation based on the minimal dilution of the donor's plasma in the recipient's blood and on the minimal count of the red blood cells of the recipient donors of this titre are perfectly safe. A margin also exists for the occasional variation of the titre in the same donor (Snyder, 1929)

Thus —

Average weight = 10 stones = 140 pounds = 2240 oz

Average volume of blood calculated at 1/20

of the weight (CO method, Haldane

and Smith) = $\frac{2240}{20}$

= 112 oz

= 112 × 28.41 c c

= 3081.92 c c

= 3082 c c approx

= 3000 c c approx

= 1500 c c

Average volume of plasma

Plasma transfused in 500 c c. of blood

The dilution of the plasma .

= 250 c c

.. = 250 in 1500 + 250

= 1 in 6 + 1

= 1 in 7.

If in the titration of the serum of the donor a 1 in 16 dilution has not reacted with 2 per cent cells then a 1 in 7 dilution will not react with 50 per cent cells in the recipient's blood, presuming the recipient to have 5 millions r b c per c mm. Even in a highly anæmic recipient having 1 million r b c per c mm the dilution of the donor's plasma, which will be nearer 1 in 8 than 1 in 7, will not react with the 10 per cent cells it will encounter. Inability of a serum which agglutinates 2 per cent cells to agglutinate suspension of higher concentration is an experimental fact detailed in section III below.

Even a donor whose serum agglutinates the r b c A and B in 1 in 16 (but not in 1 in 25) is safe enough. For quantities not exceeding 200 c.c. for an adult he is perfectly safe. The writers call this donor 'universal donor of the second choice'.

For safety these standards compare very favourably with the recommendation of Levine and Maybee (1923) in dealing with the dangerous universal donors. They recommend 'that in performing the direct matching test of Coca (*loc cit*) the donor's citrated blood be diluted with saline 2 : 10 instead of 1 : 10 before mixing with the equal volume of recipient's citrated blood. In the resulting procedure the donor's plasma in a 1 in 10 dilution (only half of the whole blood being plasma, one volume of plasma in the two parts of blood is mixed with eight volumes of saline and one volume of r b c giving a 1 in 10 dilution, if the suspension is to be used as such) reacts with the recipient's r b c concentration of which varies from 50 per cent (in a blood of normal count) to 10 per cent (in a blood having only a 1 million r b c per c mm). A 1 in 16 dilution of serum acting on a 2 per cent suspension of r b c is more effective than a 1 in 10 dilution acting on 10 to 50 per cent suspensions.

The total volume of blood calculated above is known to be a low estimate. The 'actual volume of blood in man is about 5 litres' (Short and Pratt, 1938), or 'about $\frac{1}{11}$ th of the total body-weight' (Best and Taylor, 1937). This reduces further the risk of agglutination or hæmolysis from the universal donors of the first and the second choice. For Indian donors and recipients of an average state of nutrition the lower estimate is preferable.

Incidentally, if the prospective donor is known to be O, the recommendation of Levine and Maybee (*loc cit*) can be carried out more easily by adding an equal volume of a 1 in 9 dilution of the donor's serum (instead of a 2 in 10 of citrated whole-blood suspension) to the recipient's citrated whole blood. The 1 in 10 dilution of the whole blood is really a 1 in 9 dilution of the plasma (the r b c take no part in dilution proper).

From the writers' series of 37 subjects O (Table II), from the population of Calcutta the following classification emerged —

12 conformed to the standard of the universal donors of the first choice

8 conformed to the standard of the universal donors of the second choice

11 went beyond the range of safety, their sera agglutinated r b c in a dilution of 1 in 25

- 4 were dangerous, their sera agglutinated r b c in a dilution of 1 in 50
 2 were almost certain to cause serious accidents, their sera agglutinated
 r b c in a dilution of 1 in 100

Only the first 20 were 'universal donors', the last 17 were simply donors O (to be used for recipients O)

The writers follow this classification in (i) selecting and sending donors for service from their laboratory and (ii) storing blood for the Red Cross Blood Bank which they run. Incidentally, this bank, unlike the London Scheme of Blood Transfusion Service for War (Editorial, *B M J*, 1939, Announcement, *B M J*, 1939), stores bloods of groups O A and B, not merely of O

8 '*Universal recipient*' at risk.—The risk arises from a high titre of the isonins in the blood of subjects O, A and B. As the titre of subjects A and B is not so high as that of subject O the risk with the latter is less than with the former. It always exists.

The writers apply the same 1 in 16 standard to the prospective donors of the other groups for the subjects AB

9 *Blood given to infants intramuscularly and intraperitoneally*—The blood given intramuscularly, which is not likely to increase the total volume in the body in a short time, may be given regardless of the group. Otherwise the isonins need the same consideration as they do in the blood of the donors O

Blood given intraperitoneally should be compatible or from a safe universal donor

10 *Human serum given intravenously*—The convalescent serum needs the same consideration as the blood of the 'universal donors'. The same remarks apply to the newly recommended concentrated serum and dried plasma or serum (Best and Solandt, 1940, Editorial, *B M J*, 1940, Pathological Society of Manchester, 1940, Greval, Chandra and Chowdhury, 1940)

III QUANTITATIVE RELATION BETWEEN THE ISOGENS AND THE ISONINS

1 *The quantities in system of a subject*—On the whole the isogens are relatively in excess of the requirements of the isonins. A volume of sera a or sera b is rendered inert after absorption with a fraction of an equal volume of the packed red blood cells of the appropriate isogens. Further, while most of the isonins is in the blood (or lymph) the isogen is also found in the entire cellular structure of the body

2 *Excess of red blood cells in a reaction*—In the titration the weakest dilution of a serum capable of agglutinating a 2 per cent suspension of r b c becomes inert with a 4 per cent suspension. The loss of effect in an agglutinin encountering an excess of its appropriate agglutininogen appears to be much more pronounced in hæmagglutination than in bacterial agglutination. A weak isonin is likely to be missed in grouping a subject if a thick suspension of r b c is used. Such weak isonins are often seen and their number is likely to increase when results are read within a few minutes only. In the writers' experience isonin b has been very weak at times

3 *Stability and constancy of isogens*—The isogen is not only in excess of the requirements of the isonin but also more stable and much more constant in reacting with sera of known titre. In absorption experiments, when a dilution of a constant serum and a suspension of constant r b c are used, it is quite easy to replace one suspension by another. From dried up bloodstains isonins can be extracted only on rare occasions, while isogens can be detected by an absorption test almost on every occasion.

Iso-gen A_2 and A_3 are not covered by the preceding paragraph. They react slowly and absorb the isonin incompletely.

4 *Quantity of isonins and quality of agglutinations*—A high-titre serum agglutinates the appropriate r b c almost at once. The agglutinated masses become more and more compact and centrifugal in disposition with passage of time. Ultimately they are found as a deep scarlet ring at the periphery of the drop on the slide. This is the 'centrifugal agglutination'. With very strong sera this appearance results within a few minutes.

The same serum diluted with saline brings about an agglutination which though equally distinct is neither so compact nor does it move centrifugally. Brick-red (not scarlet) masses of clumped cells appear in the centre of the drop with clear saline round them. This is the 'centripetal agglutination'.

The two types of the agglutination depend on the concentration of the isonins only.

IV THE NEED FOR A REVISION OF THE TECHNIQUE OF GROUPING BLOOD

1 *Diluted versus undiluted serum*—The isonin may at times fail to act if diluted with equal volume of saline as is being done almost universally. This appears to be responsible for 'defective groups'. The writers have not yet come across these groups. They have occasionally found an 'abnormal blood group' of another laboratory to be a case of a weak isonin. In Table II figure several sera which are likely to give trouble. If the unknown serum is to be tested undiluted with the known r b c the known serum must also be used in the same way with the unknown r b c so that final mixture of r b c and serum have a constant density in both the tests.

The 'cold agglutination' is said to be avoided by the dilution of the serum. The writers take their sera out of the refrigerator and keep them at room temperature for half an hour. They have not seen any 'cold agglutination'.

2 *The time*—The time usually allowed at least for the slide method is not enough. A_2 and A_3 are likely to be missed. In India the preparations dry up rapidly at times and further shorten the time of reaction. A moist-chamber must be used and the reaction observed for 30 minutes. There is no possibility of a false reaction appearing merely with the passage of time.

3 *Whole blood and plasma*—The use of whole blood, in saline and even in a sodium citrate solution, in testing the r b c for the isogen is faulty. Clotting of

blood occurs and looks like agglutination of r b c. The unknown r b c must be washed clean of fibrin. The same remarks apply to the testing suspension. The use of plasma from centrifuged whole blood (instead of the serum) is open to the same objection. These methods are not recommended by any one but are tolerated. They should not be tolerated. Blood transfusion is an operation which is likely to kill on the spot if full precautions for safety have not been taken. Short cuts in testing for group and matching bloods fall short of full precautions.

V A REVISED TECHNIQUE OF GROUPING AND MATCHING BLOOD

1 *The apparatus* —The apparatus required has been detailed in the technique of titration and is shown in Plate IX.

The known serum is derived from healthy donors of blood and is selected. In a 1 in 5 dilution it should agglutinate an equal volume of a 2 per cent suspension of known appropriate cells within a minute. An undiluted serum of this potency generally produces a centrifugal agglutination in about 5 minutes. Some strong sera in a fresh state cause hæmolysis of the cells and need inactivation or storage for several days.

The known r b c are also derived from healthy donors of blood. Each serum is tested against three samples of appropriate and inappropriate cells. The inappropriate cells should not be agglutinated. All samples of cells B should be agglutinated with the same or almost the same intensity and speed. Occasionally a sample of cells A may be found to respond with less speed and/or less intensity. Another sample then is tried in its place before the serum is accepted as a testing serum.

The testing serum is used in testing the unknown cells and the known cells are used in testing the unknown serum. For blood transfusion always and for other purposes whenever possible both the r b c and the serum should be tested. In determining group from isogen only, as is done in the case of stains of blood, some findings have to be ignored.

The workers in the laboratory should know their own group. Most subjects can tell the group of most other subjects after watching the reactions of the latter's cells and serum against their own.

The cells and sera are preserved without antiseptics in a refrigerator and renewed once a week or so. For the samples used for testing, surgical asepsis is all that is necessary, while for those used for injecting animals, for experimental work, bacteriological sterility must be maintained. A few sera are also kept frozen.

2 *The unknown cells and sera* —From each subject under test 1 c c to 5 c c of blood are drawn from a vein with a syringe. One half of the blood is squirted, for r b c, into a tube (or tubes) containing an equal volume of 1.5 per cent of sodium citrate in normal saline or ten volumes of normal saline, and the other half, for serum, into a dry tube. The tube for the r b c is shaken and centrifuged. The supernatant fluid is replaced by normal saline at least twice. The tube for serum is

left undisturbed until a clot has formed, contracted and ejected a clear serum. A part of the deposit from the centrifuged tubes is measured and removed to make a 2 per cent suspension. The tube with the clot is also centrifuged if necessary and the clear serum transferred to another tube.

The small quantity of blood is taken from a recipient and large quantity from a donor. From the latter's blood stocks of known cells and sera (after the test, when the group is known) are prepared and kept. Whole blood added to ten volumes of saline (with sterile precautions) and shaken can be left in a refrigerator and washed when necessary. Two or three tubes are prepared for a single donor. They keep for well over a week. Similarly, a stock of clear serum is also prepared from the same donor. The serum keeps longer than r b c.

When only a sample of uncitrated whole and, therefore, clotted blood is available, the serum is removed as before and the clot shaken in normal saline to liberate r b c. The latter are separated from particles and washed once.

Enough r b c and serum can be obtained with care even from a few drops of blood clotted from a finger, toe or lobe of the ear, as is done in taking a specimen for a Widal test or from infants and young children.

3 *The grouping test proper—for determining the isogens A and B in the unknown r b c*—An old slide is preferred to a new one. Fluid on such a slide can be spread more easily. The obverse is divided by a line drawn with a glass-marking pencil into left and right halves (opposite worker's left and right, commencing with left as in writing). On the reverse, along the lower border, are inscribed (i) the distinctive initials or number of the specimen, at the extreme left, (ii) 'a' in the middle of the left half, and (iii) 'b' in the middle of the right half. In the left half is deposited one drop of the testing serum a with a calibrated capillary finger pipette (hereafter called the finger pipette). Similarly, in the right half is deposited one drop of the testing serum b. To each drop is added one drop of the suspension of the unknown r b c with a finger pipette. The cells and the serum are mixed and spread over an area about $\frac{3}{4}$ " in diameter with the special rod moving clockwise and then counter-clockwise, the same number of times for each movement and for each mixture. The two ends of one rod mix and spread the mixture on one slide. The finished slide is left in the moist-chamber for observation after 5 minutes, 15 minutes and 30 minutes. Four reactions are possible: (i) if only a agglutinates, the blood is A, (ii) if only b agglutinates, the blood is B, (iii) if both a and b agglutinate, the blood is AB, and (iv) if neither a nor b agglutinates, the blood is O. The slide falling short of a full agglutination (the clumps without sharp margins and saline between them not perfectly clear) is re-tested with 2 drops of the testing serum. Generally it is the left side.

An alternate method for determining the isogens in the r b c—If the testing sera do not conform to the standard, i.e. a 1 in 5 dilution of them does not agglutinate the appropriate cells almost immediately, then a strong serum ab (from subject O) is also used as a testing serum. The slide is divided on the obverse into three compartments. On the reverse, along the lower border, are inscribed (i) the distinctive initial or number of the specimen at the extreme left,

(*ii*) 'ab' in the middle of the compartment on the left, (*iii*) 'a' in the middle of the compartment in the middle and (*iv*) 'b' in the middle of the compartment on the right. Drops of testing sera are deposited in their proper compartments. Drops of the r b c suspension under test are added, mixed and spread as before. An extra rod will be needed. If serum ab agglutinates, the blood is A, B or AB. It cannot be O. This extra test excludes a false reading of O due to a weak isonin in the testing serum. This exclusion is very important in transfusion. Evidence is accumulating to show that all over the world slowly reacting A's have been mistaken for O's, with disastrous results. This is quite likely to occur if b in the same subject is also weak and has been further weakened by dilution of the serum. Isonins in sera ab are generally stronger than those in sera a and b.

For determining the isonins in the unknown sera—The slides are divided into two halves as before and inscribed on the obverse, along the lower border with (*i*) distinctive initials or number of the specimen, (*ii*) 'A' in the middle of the left half and (*iii*) 'B' in the middle of the right half. In the left half is deposited with a finger pipette one drop of the known r b c suspension A and in the right half with another finger pipette one drop of the known r b c suspension B. To each drop is added one drop of the unknown serum. The cells and the serum are mixed together, spread and left in the moist-chamber for observation after 5 minutes, 15 minutes and 30 minutes. Four reactions are possible (*i*) if A alone is agglutinated, the serum is a, (*ii*) if B alone is agglutinated, the serum is b, (*iii*) if both A and B are agglutinated, the serum is ab, and (*iv*) if neither A nor B is agglutinated, the serum is o (not O, of subject AB). The side falling short of a full agglutination is re-tested with 2 drops of the unknown serum.

The determination of the isogens and isonins is further explained in the attached plan (see page 245).

For determining the isogens A_2 and A_3 —The writers' experience of A_2 is limited. They have studied slowly and/or incompletely agglutinating cells A from time to time by absorption by the following process. From half the suspension a packed volume of r b c is obtained in a quill-tube. This volume is mixed with two volumes of the test serum a and left at room temperature for 30 minutes, in the refrigerator for 30 minutes and at room temperature again for 30 minutes. The quill-tube is placed in an almost horizontal position to allow full contact between the cells and the serum during absorption. The tube is next centrifuged and the absorbed supernatant clear serum removed. One drop of the absorbed serum is tested with the original suspension and another drop with a suspension of normally agglutinating r b c A. If the first drop does not agglutinate while the second does, the slowly and/or incompletely agglutinating cells are cells A_2 .

The absorption may be incomplete. In that case the incompletely absorbed serum is absorbed further with a quarter of its volume of packed cells and re-tested.

All slowly and/or incompletely agglutinating cells A do not appear to be A_2 .

Of the newly described A_3 (Wiener, *loc cit*) the writers have no experience.

The subgroups of A have no significance in blood transfusion other than that A_2 and A_3 are likely to be mistaken for O, and A_2B or A_3B for B. Their significance in forensic medicine lies in the fact that A ($= A_1$) is dominant to A_2 which is dominant to A_3 . The writers, however, consider that pending further clarification of the situation the differentiation between A_2 and A_3 for medico-legal purpose would be unwise. The difference may be one of quantity only and its inheritance not so rigid as that of a difference of quality.

It is suggested that all slowly reacting A's should be designated slow A's.

The essential features of the technique—These are two (i) the serum (testing or unknown) should not be deficient relatively to the r b c and (ii) the time allowed should be at least 15 minutes and extended to 30 minutes in case of a suspicious reaction. The error in the density of the r b c suspension, if error there must be, should be on the side of a lower density not a higher density. If a calibrated capillary finger pipette is not available an uncalibrated capillary pipette may be used but the drop of the serum as seen by the eye on the slide should not be smaller than that of the r b c suspension, it may be bigger. The suspension under these circumstances is deposited alongside the other drop, not added to it. Even in respect of time the error if any, should be on the side of allowing more time. The preparations kept longer than 30 minutes do not give false reactions.

Using this technique no defective groups or anomalous reactions have been found in bloods of healthy subjects although a sharp look out has been kept for them for 6 years. Even in the bloods of diseased subjects incompatibility within the same group or other anomalous reactions due to morbid causes, have been encountered on rare occasions only. Further following this technique this laboratory has run a blood transfusion service for Calcutta for the last 16 years. Bloods of donors and recipients of all communities in Calcutta European and Indian have been grouped (Greal and Chandra 1940).

Suggestions for field workers—Field workers can easily carry all the glassware used together with a hand centrifuge. The capillary pipettes are sterilized with dry heat after dipping their lower ends in a 1.5 per cent sodium citrate solution in saline (the solution used for mixing with whole blood, for r b c). Blood taken in a pipette so prepared does not coagulate at once. By attaching the pipette by plasticin to one end of a short length of glass tubing carrying at the other end a rubber teat one drop of blood can be very easily added to $\frac{1}{2}$ c.c. of normal saline in a tube. The drop of blood equals half a drop of r b c deposit which when added to the 25 drops contained in the $\frac{1}{2}$ c.c. of saline yields a $\frac{1}{2}$ in 25 (approx.) or 1 in 50 or 2 per cent suspension. The tube is shaken. The level of the suspension (top of the meniscus) is marked and the tube centrifuged. The clear supernatant fluid is poured off and replaced by fresh saline. The tube is shaken and a 2 per cent suspension of r b c almost free from fibrin obtained.

For lining the inner surface of the lower end of the capillary pipettes liquid paraffin may be used instead of sodium citrate. The pipettes must be sterilized as before.

The pipettes after use are dropped in a cylinder containing 10 per cent hydrochloric acid (the fluid should effervesce energetically when dropped on cement floor) and glass-wool at the bottom. Next day they are washed in running water (boiled if they have been paraffined), dried and examined individually to make sure that the lower calibrated end is unbroken. The pipettes can be washed and used again and again. Obstruction to the flow of fluid up the pipette is overcome by the test or even by opening and closing the end by the finger repeatedly and quickly, several times.

4 *Direct matching tests—within the group*—Having decided that the prospective donor and recipient belong to the same group, a drop of the *r b c* suspension of the donor is mixed with a drop of the recipient's serum, this is done on the left half of a slide marked on the reverse *r + D*. Similarly, a drop of the *r b c* suspension of the recipient is mixed with a drop of the donor's serum, this is done on the right half of the same slide marked on the reverse *d + R*. The capital letters represent the isogen and the small letter the isonin of the donor and the recipient respectively. There should be no agglutination. This test is especially important in the case of a second transfusion from the same or even a different donor. The first transfusion although within the group may produce antibodies, unconnected with A and B, which may react with their appropriate antigen in the second transfusion. A case illustrating this acquired incompatibility has been reported (Neter, 1936).

Incompatibility within the group without a previous transfusion, probably due to a morbid state, is known (Lloyd and Chandra, 1933). The possibility of a naturally occurring anti-M body giving rise to such an incompatibility also exists (Wiener, *loc cit*).

The formation of anti-M and anti-N bodies after a previous transfusion with blood compatible with respect to O, A and B but not M and N is a new suggestion (Greval *et al*, 1939, 1940).

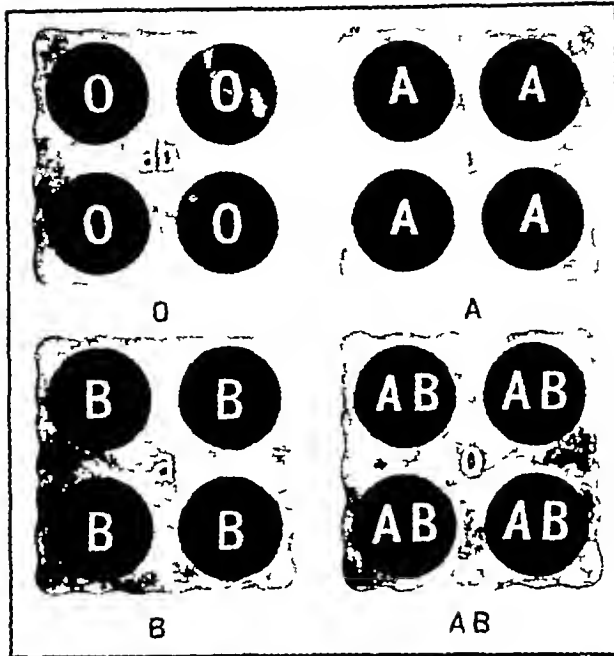
Donors O for other groups and donors of other groups for recipient AB—A 'safe' universal donor must be used in emergency without any preliminary tests. But when such a donor is being used for other groups, for other reasons, his serum *in a 1 in 16* dilution and *r b c* must also be directly matched with the recipient's *r b c* and serum, for the same reasons for which direct matching is done between the donor and recipient of the same group. Serum of a prospective recipient of group A, B or AB may due to morbid states, agglutinate prospective donor's *r b c*, containing the recipient's own isogen (Lloyd and Chandra, *loc cit*). A safe universal donor then may prove to be the only compatible donor.

Similarly, a *1 in 16* dilution of the serum of the prospective donors O, A and B must be tested on the *r b c* of a prospective recipient AB.

Matching sera, leucocytes and platelets—The writers observe on a slide in a moist-chamber the mixture of the serum of the prospective recipient and donor after 30 minutes' incubation at 37°C. The slide has on it, in three compartments, (i) recipient's serum one drop + donor's serum one drop, (ii) recipient's serum 2 drops and (iii) donor's serum 2 drops. (i) and (iii) are controls. There should be no precipitate or increase in opalescence.

PLATE VIII

A diagram of blood groups



Jansky

I

II

III

IV

Moss

IV

II

III

I

New

O

A

B

AB

A and B are the isogens

a and b are the corresponding isonins

The group is named after the isogens

The four groups indicate the only four possibilities compatible with life, in which the isogens and the isonins can co exist in the same subject. In a 'defective' group an isonin which can exist compatibly with life is absent (e.g. O, a, A, o)

Further division of A into A_1 and A_2 (and into A_1B and A_2B) increases the number of groups to six

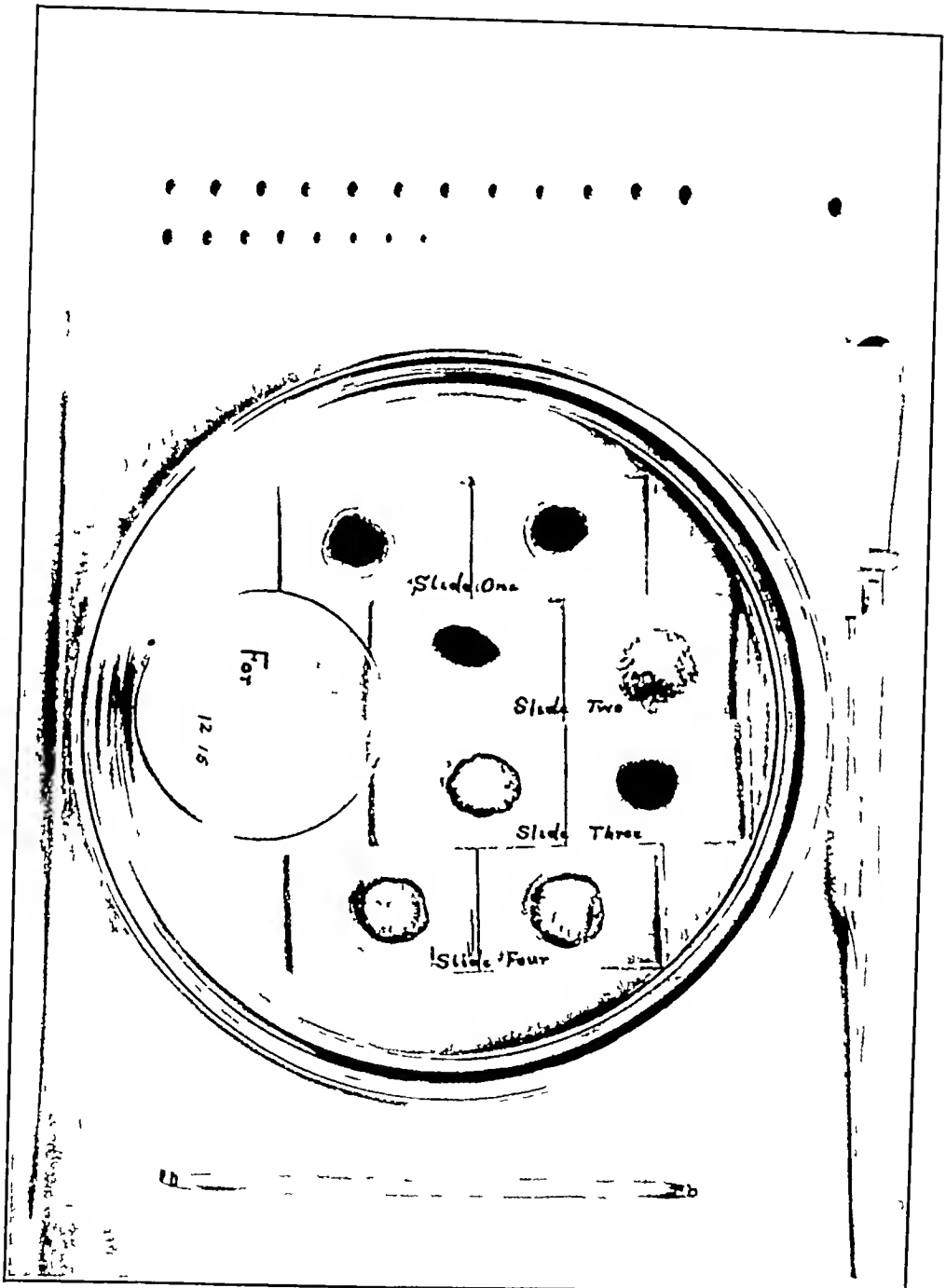
Quite unrelated to A and B are the hæmogens (not isogens) M and N. They occur as M, N, or MN in all subjects of all groups. No subject is free from them. They differentiate three types in each group and thus make possible 12 descriptions of blood, if only the original four groups are considered, or 18 descriptions, if sub groups with A_1 and A_2 are also considered. A blood may be —

OM	ON	OMN
AM	AN	AMN
BM	BN	BMN
ABM	ABN	ABMN

Very recently A_3 has been added to the list of isogens. A_2 and A_3 together with the slowly reacting A not differentiated by absorption constitute 'slow A's' of the writers. They have no significance in blood transfusion. In forensic medicine too they are ignored by many workers because of difficulties of technique. M and N are easily determinable and have received due recognition in forensic medicine all over Europe and America.

PLATE IX

A photograph of apparatus and naked-eye appearance of reactions.



The slides may be 1 or 3 of the plan for determining isogens and isonins respectively. On the dotted line after 'For' is written 'cells' or 'serum'.

If for cells, slide one indicates O, slide two B, slide three A and slide four AB.

If for serum, slide one indicates o (from subject AB), slide two b (from subject A), slide three a (from subject B) and slide four ab (from subject O).

The special apparatus consisting of wire gauge (L S Starrett Co., U S A), moist-chamber, calibrated pipettes (finger and teat, point fitting hole No 58 of the wire gauge) and stirring rod is shown.

The naked eye appearance is distinct.

PLANS OF GROUPING AND MATCHING BLOODS

1 Determination of the isogons

No	a	b
----	---	---

The slide is marked for the known serum
The r b c suspension is unknown The agglutinator indicates the agglutinated

or

No	ab	a	b
----	----	---	---

The plan is adopted when the titre of the testing sera does not come up to the standard

2 Determination of the isonins

No	A	B
----	---	---

The slide is marked for the known r b c
The serum is unknown The agglutinated indicates the agglutinator

3 Matching of cells and sera

r + D	d + R
-------	-------

r = Recipient's serum

R = „ r b c

d = Donor's serum

D = „ r b c

There should be no agglutination

4. Matching of sera

d + r	d + d	r + r
-------	-------	-------

Double volume of each serum is used for controls of volume and pre-existing opalescence
There should be no precipitate or increase in opalescence

As an additional precaution the writers recommend a scratch test on the skin, whenever possible, especially in the case of a second transfusion from the same donor

A complement-fixation test has been recommended and may be undertaken. The writers suggest the following procedure. To one volume of a 1 in 5 dilution of one serum are added one volume of complement containing 2 m h d and one volume of a 1 in 5 dilution of the other serum. The three constituents are incubated for 30 minutes at room temperature and 30 minutes at 37°C. One volume of sensitized r b c suspension is then added and incubated at 37°C for 30 minutes. If the recipient's serum is anticomplementary it is diluted further. A donor whose serum is anticomplementary is rejected. The writers do not perform the test as a routine. Their hæmolytic system is prepared in accordance with Method No. 4 of the Wassermann Test of the (British) Medical Research Committee (now Council) (1918).

Tests for the incompatibility of the leucocytes and platelets (Snyder, *loc cit*) need not be performed.

The direct matching tests are further explained in the attached plan.

SUMMARY

1. Red blood cells (and subjects) are designated cells (and subjects) (1), A (including A₂ and A₃ and slow A), B and AB. The sera are a (from subject B), b (from subject A) and o (small letter, from subject AB). Serum a₁ (not acting on A₂) is obtained after absorption with A₂. Serum a₂ (acting on A₂ only) may be present abnormally. *Grouping* means determining O, A and B. *Typing* means determining M and N with anti-M and anti-N fluids. Isohæmagglutinogens and isohæmagglutinins may be called isogens and isonins, and M and N hæmogens α , β and 'factors' are not necessary.

2. A method of making many dilutions in one tube for titrating isonins is described. Observations are made on the titre, including the existence of the dangerous 'universal donors'.

3. There is more isogen than isonin in the body. Quantitative differences in concentration of isonin yield qualitative differences in isohæmagglutination (centrifugal and centripetal).

4. In the technique now used (i) concentration of testing sera is reduced and (ii) enough time is not given for reaction. Slow A's and weak b's are at times missed.

5. A new technique is described in detail, including suggestions for field workers.

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TABLE I
Showing the scheme and arithmetic of dilutions

	Drops of serum added	Drops of serum already present	Initial volume in drops	Ultimate volume in drops	Strength
To 1 cc of normal saline	{	<i>Nil</i>	50	51	1 in 51 = 1 in 50
		1	49	50	2 in 50 = 1 in 25
		2	48	49	3 in 49 = 1 in 16
		3	47	48	4 in 48 = 1 in 12
		4	46	47	5 in 47 = 1 in 9
To the same dilution (after testing 2 drops)	{	5	45	46	6 in 46 = 1 in 8
		6	44	45	7 in 45 = 1 in 7
		7	43	44	8 in 44 = 1 in 6
		8	42	43	9 in 43 = 1 in 5
		9	41	42	10 in 42 = 1 in 4
		10	40	41	11 in 41 = 1 in 4 also
		11	39	40	12 in 40 = 1 in 3
Or to 1 in 9 dilution	3	5	45	48	8 in 48 = 1 in 6
From volume left after using 2 drops discarded 17 drops and to the remaining dilution	7	5*	29	36	12 in 36 = 1 in 3

from the ultimate volume 2 drops are used in the test, that is why the initial volume of the next dilution measures 2 drops less

The dilutions are really weaker, that is why in most cases approximation has been made towards the figures indicating weaker dilutions (7 in 45 has been taken to be 7 in 49 not 7 in 42)

* Three drops of serum are discarded in the 17 drops of the last dilution, thus $\frac{17 \times 8}{48} = \frac{17}{6} = 3$ (approx) These 3 drops subtracted from the 8 drops of the last dilution leave 5

In preparing the dilution for absorption the dilution indicated by the table is made, tested and adjusted by adding more serum, if necessary In titrating sera for selecting universal donors only the upper three dilutions are concerned, in them the error is negligible In determining the titre for a general record the error in the lower dilutions is immaterial

TABLE II

Giving titres of 37 sera ab, 38 sera a and 31 sera b

	REACTION IN DILUTION OF 1 IN —												Under 4
	100	50	25	16	12	9	8	7	6	5	4		
Sera ab, 37,													
Component a,													
i Sera giving sharp reaction in one dilution 7	1	1	3	0	1	0	0	0	0	0	1	0	
ii Sera giving increasing reaction in two dilutions, 23,													
(i) initial* reaction, \pm ,	1	;	4	2	5	5	0	2	0	0	1	0	
(ii) completed† reaction, +,	0	1	3	4	2	5	5	0	2	0	0	1	
iii Sera giving increasing reaction in three dilutions, 7,													
(i) initial reaction, \pm ,	0	0	3	3	1	0	0	0	0	0	0	0	
(ii) completed reaction, +,	0	0	0	0	3	3	1	0	0	0	0	0	

* Required for ascertaining the suitability of donors O for recipients A, B and AB and of donors A and B for recipients AB Even \pm in a given dilution rejects donor

† Required for determining the dose of sera ab, a and b for absorption tests Maximum reaction is arrived at in reading results and therefore used in choosing dose

TABLE II—*concl'd*

Component <i>b</i> ,	REACTION IN DILUTION OF 1 IN —												Under 4
	100	50	25	16	12	9	8	7	6	5	4		
i Sera giving sharp reaction in one dilution 5,	1	0	1	0	2	1	0	0	0	0	0	0	
ii Sera giving increasing reaction in two dilutions, 27,													
(i) initial reaction, \pm ,	0	0	3	3	1	7	4	1	1	0	0	2	
(ii) completed reaction, +	0	0	0	3	3	1	7	4	1	1	0	2	
iii Sera giving increasing reaction in three dilutions, 5,													
(i) initial reaction, \pm ,	0	0	1	2	0	1	0	1	0	0	0	0	
(ii) completed reaction, +,	0	0	0	0	1	2	0	1	0	1	0	0	
Sera a, 38,													
i Sera giving sharp reaction in one dilution, 9,	0	4	3	0	0	1	1	0	0	0	0	0	
ii Sera giving increasing reaction in two dilutions, 22,													
(i) initial reaction, \pm ,	0	0	5	1	3	4	5	2	1	1	0	0	

(ii) completed reaction, +

iii Sera giving increasing reaction in three dilutions 7

(i) initial reaction, \pm .

(ii) completed reaction, +

Sera b, 31,

i Sera giving sharp reaction in one dilution 9

ii Sera giving increasing reaction in two dilutions, 15

(i) initial reaction \pm

(ii) completed reaction, +,

iii Sera giving increasing reaction in three dilutions 7

(i) initial reaction, \pm

(ii) completed reaction, +,

0	0	0	0	0	1	1	1	2	1	1	0
0	0	1	0	1	1	1	0	0	1	0	0
0	0	0	0	1	0	1	1	1	0	0	1
0	1	1	1	1	1	1	1	0	0	0	0
0	0	0	3	1	1	1	1	0	2	1	0
0	0	0	3	1	1	1	1	1	2	1	1
0	0	1	2	2	0	0	0	0	2	0	0
0	0	0	0	1	2	2	0	0	0	0	2

A specimen page from record of titration of sera

Serial number of serum	REACTION, IN 5 MINUTES WITH CELLS --		Group	REACTION WITH APPROPRIATE R B C OF DILUTIONS 1 IN --													REMARKS
	A	B		100	50	25	16	12	9	8	7	6	5	4	Under 4		
111	+	+	-	-	-	-	-	-	-	+	+	+	+	Under 4	{ Isoton very weak, might have been missed with dilution of serum and in reading results in less than 15 minutes.		
112	+	+	-	-	-	+	-	-	-	+	+	+	+				
113	+	-	-	-	-	-	-	-	-	+	+	+	+				
114	+	-	-	-	-	-	-	-	-	+	+	+	+				
115	-	+	A { b in 5 minutes b in 15 "	-	-	-	-	-	-	-	-	-	-	-	{ Isoton very weak, might have been missed with dilution of serum and in reading results in less than 15 minutes.		
116	+	-	B { a in 5 minutes a in 15 "	-	-	-	-	-	-	-	-	-	-	-			
117	-	-	AB No isonun	-	-	-	-	-	-	-	-	-	-	-			
118	-	+	A { b in 5 minutes b in 15 "	-	-	-	-	-	-	-	-	-	-	-	{ Isoton b missed with dilution		
119*	+	±	O { a in 5 minutes a in 15 ; b in 5 ; b in 15 ;	-	-	-	-	-	-	-	-	-	-	-			

* No 119 includes discarded cases in the beginning of the series and cases AB possessing no isonun.

PROTEIN FRACTIONS AND OTHER PHYSICAL PROPERTIES OF HYDROCELE FLUID

BY

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THE present studies refer solely to hydroceles believed to be definitely of filarial origin. The characteristics of such hydroceles are as follows. In the large majority of cases fluid begins to collect quite insidiously on one or both sides. It may appear quite suddenly after an inflammatory attack of the testes and epididymis and subsequently undergo absorption. After successive attacks the fluid accumulates with a gradual increase in the size of the hydrocele. The size of filarial hydrocele varies, the average being of the size of a tennis ball. The swelling is generally painless. On aspiration the fluid is clear and serous in colour and shows varying amounts of fibrinogen, as seen by the formation of white film and coagulation of the fluid on standing for a few minutes. In 2 to 3 per cent the fluid is opaque and milky and with microfilariae in them. The fluid in long-standing cases is generally full of cholesterol crystals. Normally the fluid is sterile and shows ordinary tissue elements and inflammatory cells.

So far as the authors are aware very little work seems to have been done on the protein fractions and other physical properties of lymph obtained from tropical hydrocele*. This has led us to investigate some of its properties, which might enable us to throw some light on the subject.

* For protein fractions of non tropical hydroceles see Wells (1925) and Paton (1906)

EXPERIMENTAL PROCEDURE

Estimation of proteins—The method used by us was essentially the micro-refractometric method of Robertson (1915). Estimations were made with the help of a Dipping Refractometer (Zeiss).

A saturated solution of ammonium sulphate at 21.5°C containing about 54 g of ammonium sulphate per 100 c.c. of the solution was used as the precipitating electrolyte. The experiments were performed at room temperature. The fraction precipitated by 50 per cent saturation is taken as the total globulin, while the amount separated with 33 per cent saturation is regarded as the euglobulin fraction, and the difference between the percentages of these two is the percentage of the pseudoglobulin.

Surface tension was determined by the du Nouy's apparatus in the way indicated by Chopra and Choudhury (1928).

Buffer action—The pH values were determined electrometrically by the quinhydrone calomel chain. The readings were always taken within 3 minutes. The results are given in Tables I and II.

TABLE I
Protein fraction and sugar content

Serial number	Albumin, per cent	Euglobulin, per cent	Pseudoglobulin, per cent	Case number	Sugar per cent
1	3.15	0.27	2.30	A	0.114 (R)
2	3.01	0.27	2.44		0.122 (L)
3	3.00	0.15	1.77		
4	3.35	0.08	0.93	B	0.110—(23-7-40)
5	5.71		0.18		0.120—(30-7-40)
6	2.60		0.61	C	0.100 (R)
7	2.56	0.91	1.18		0.084 (L)
8	3.78		0.76	D	0.016 (sugar value estimated 36 hours after the collection)
9	3.70	0.67	0.61		
10	2.11	1.02	1.77		
11	3.61	1.11	0.91		
12	2.38	0.86	1.84	E	0.092
13	3.00	1.34	0.67	F	0.121—(19-7-40)
14	3.90		0.69		0.075—(23-7-40)

TABLE I—*concd*

Serial number	Albumin, per cent	Euglobulin, per cent	Pseudoglobulin, per cent	Case number	Sugar, per cent
15	1.87	0.80	1.70	G	0.130
16	4.00	1.44	0.58	H	0.115
17	2.53	1.50	1.35	I	0.130
18	4.23	1.37	0.55	J	0.080
19	5.49	0.21	0.52	K	0.096

TABLE II

Buffer action

Serial number	Original pH	pH after addition of equal amount of N/10 HCl	pH after adding equal amount of N/100 HCl	Surface tension in dynes *
1		2.66	7.04	52.3
2	7.21	2.64	6.92	48.5
3	7.27	2.79	6.89	50.5
4	7.19	2.39	6.95	47.4
5	7.18	2.75	6.82	50.5
6	7.17	2.73	6.82	47.4
7	7.20	2.60	6.79	47.9
8	7.22	3.09	7.00	47.9
9	7.31	2.31	6.76	48.3
10	7.17	3.34	6.88	48.8
11	7.31	2.50	6.85	50.5
				49.7
				50.6

* Surface tension values although included in this table are from a different series of patients

From a perusal of Table I it will be seen that the total protein values show a great variation ranging from 3.21 to 5.71. In 8 cases the pseudoglobulin fraction is rather high, while in 6 cases the euglobulin is also high. In these 6 cases no time was allowed for the separation of the fibrin. It is, however, known that fibrin is precipitated by $\frac{1}{2}$ ammonium sulphate and this explains the high value of euglobulin found in these cases. The amount of sugar has been determined in some samples and its amount varies from 0.016 per cent to 0.122 per cent as will be seen from Table II. Two very peculiar facts are observed from a study of the sugar content. In the same patient the right and left hydrocele fluids show different amounts of sugar and the amount of sugar in the fluid of a patient is different on different dates. It is well known that the sugar content depends upon the nature of the food taken by the patient and possibly also upon the state of the inflammation. The findings are, however, very interesting and will be discussed in a later paper. The amount of albumin found in the fluids is neither low nor high compared to normal sera. The total protein content is also not very low. This is interesting in view of the fact that the patients having hydrocele are otherwise normal. There is no disturbance of the metabolism or any manifestation of other pathological symptom. It has been shown by Chopra, Mukherjee and Rao (1934) that the physical and chemical characters of the blood in filariasis patients do not change to the same extent as are found in certain protozoal diseases such as kala-azar, syphilis, etc. where the changes are more marked. It is, therefore, probable that the high concentration of the protein in the hydrocele fluid is not derived from the blood stream in which case the blood would have been appreciably poorer in protein content. Peters and van Slyke (1931) favour the view of Starling, that where the protein content of tissue fluids is not high but only comes up to as much as 0.05 to 0.35 per cent, there is probably no change in capillary permeability. Where the protein content reaches a higher value (up to 1 per cent nearly) as in angio-neurotic oedema there are reasons to believe that capillary permeability has changed. A still greater concentration (beyond 1 per cent) has been explained by Schade, Claussen and others (1926) as being derived mainly from disintegrated cellular tissue and only partly from the proteins in the blood stream. In support of this they showed that purulent material from abscesses even if it is freed from cells by centrifugation may contain as much as or more protein than serum. In the light of these observations it is reasonable to conclude that the hydrocele fluid is an inflammatory exudate.

The low value for the surface tension is difficult to explain. In a previous paper (Chopra and Choudhury, *loc cit*) it was pointed out that euglobulin is responsible for the greater lowering of surface tension in sera from kala-azar patients. But in hydrocele fluids there is no such increase of euglobulin. However, in course of our experiments with the fluids we always noticed a distinct colour of the fluids which suggests the presence of bile salts which are known to diminish the surface tension. Cholesterol also depresses the surface tension to a certain extent, but the amount of cholesterol generally present in these fluids is not sufficient to account for the diminution in surface tension. It is also possible that some surface active substances are produced as a result of the local inflammation.

From Table II it will also be seen that the pH and buffer action are also slightly lower than that of sera. Normal or pathological sera in kala-azar, malaria, filaria, etc. have all got slightly higher buffering capacity. This is in keeping with the observation that all exudates have low buffering capacity, but in our cases it is not so low as in other exudates. This slight diminution may possibly be due to the lower protein content of the fluids.

SUMMARY AND CONCLUSIONS

The protein fractions of hydrocele fluids have been estimated following the method of Robertson. The results show that the fluids belong to the class of exudates.

The buffer action and surface tension of these fluids are low. Sugar in varying amounts is present in all the hydrocele fluids examined.

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STAPHYLOCOCCI IN VACCINE LYMPH

BY

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(From the King Institute of Preventive Medicine, Gundy)

[Received for publication, November 22, 1940]

IN laying down standards of purity for calf lymph, the significance of *staphylococci* has been vague. There has been no agreement as to what extent the presence of *staphylococci* should be restricted in a given batch of lymph to be issued for field use. While the Ministry of Health in England have laid down in the Therapeutic Substances Act of 1925 as a minimum standard of purity of vaccine lymph, 5,000 organisms per c c, no question has been raised as regards their pathogenicity. The Office Internationale d'Hygiene Publique appointed in 1932 a Committee on vaccination whose questionnaire to various vaccine lymph experts did not elicit any opinion which may be considered unanimous because the proportion in which these organisms occurred in lymph varied within wide limits in different institutes in spite of all precautions in the manufacture. The Committee also published in 1933 a report by Dr. Davide of Stockholm on the biological and pathogenic characters of several strains of *staphylococci*. All these strains of *staphylococci* were of human origin, both from pathological lesions and from normal skin. There is no record of a similar study on *staphylococci* isolated from calf lymph.

This note is based on a study of *staphylococci*, with special reference to *S. aureus*, isolated from calf lymph during a period of three and a half years at the King Institute, Gundy. Several strains of *S. aureus* and *S. albus* were isolated from different batches of calf lymph soon after manufacture and before the lymph had more than one week's contact with 50 per cent glycerine, and also from lymph stored at a temperature below 0°C for more than three months.

For isolation of *staphylococci* 0.1 c c of lymph in a 1 in 5 dilution was inoculated in 10 c c of glucose broth. After 48 hours' incubation at 37°C, the broth culture was plated on agar plates and incubated for 24 hours. From the plates, several colonies of *staphylococci* were subcultured. The pathogenic character of the organisms was determined by the presence of certain properties described by various workers from time to time as indicative of pathogenicity, viz. plasma coagulation, hæmolysis and mannite fermentation. The technique of Fisher (1936) was employed.

for the plasma coagulation test and that of Thompson and Khoraso (1937) for hæmolysis and mannite-fermentation tests. The power of production of exotoxin and its pathogenicity to rabbit skin was determined after the method of Parker (1924). In all 373 strains of *S. aureus* and 80 strains of *S. albus* obtained from lymph soon after manufacture and 132 strains of *S. aureus* isolated from lymph stored below 0°C for more than three months were studied. They are divided into three groups. The analysis of these groups is given in Tables I, II and III —

TABLE I.

Analysis of group I—373 strains of S. aureus

Nature of tests	Number positive	Percentage
Toxicity to rabbit skin	2	0.53
All three tests positive	21	5.63
Plasma coagulation alone	32	8.57
Hæmolysis alone	37	9.91
Mannite fermentation alone	58	15.55
All tests negative	222	59.51

TABLE II

Analysis of group II—80 strains of S. albus

Nature of tests	Number positive	Percentage
Toxicity to rabbit skin		
All three tests positive		
Plasma coagulation alone	26	32.5
Hæmolysis alone	6	7.5
Mannite fermentation alone	13	16.25
All tests negative	35	43.75

TABLE III

Analysis of group III—132 strains of *S. aureus* isolated from lymph stored not less than three months

Nature of tests	Number positive	Percentage
Toxicity to rabbit skin		
All three tests positive		
Plasma coagulation alone		
Hæmolysis alone	10	7.5
Mannite fermentation alone	19	14.34
All tests negative	103	78.03
<i>N.B.</i> —Three tests 1 Plasma coagulation 2 Hæmolysis 3 Mannite fermentation		

In Table I under group I, out of 373 strains of *S. aureus*, only 21 strains or 5.63 per cent were simultaneously positive to three tests, viz plasma coagulation, hæmolysis and mannite fermentation. Two hundred and twenty-two or 59.51 per cent of the strains were entirely negative to all tests. The remaining 128 strains gave positive reactions to one or two only of the tests. Two strains, i.e. 0.53 per cent, gave a positive toxicity test to rabbit skin, but they did not give this reaction to monkey skin (*M. radiata*). These two strains were from among those 21 strains which gave the three tests positive. Among others none gave a positive toxicity reaction when tested on rabbit skin.

In Table II under group II *S. albus*, it will be seen that not one strain out of the 80 has given all the three tests simultaneously positive, 43.75 per cent were negative to all tests, the rest were positive to one or two tests only, not one giving a positive toxicity reaction on rabbit skin.

In Table III under group III, out of 132 strains of *S aureus* isolated from lymph after storage for over three months, not one strain was positive to all the three tests 78.03 per cent were negative to all tests. Again, it is significant to note that all the strains were negative to the plasma coagulation test to which reaction some workers attach considerable importance in determining the pathogenic character. Not one strain was positive to the toxicity test on rabbit skin, although two strains in this group were isolated from the same two batches of lymph from which two toxic strains under group I were isolated. It is thus clear that the toxic strains of *S aureus* with power to produce exotoxin capable of producing lesions on rabbit skin do occur though very rarely, only 0.53 per cent in this group of 373 strains. These two strains, when tested for toxicity on monkey's skin, gave negative results on repeated trials. It is also shown that these organisms do not retain their toxic property when isolated from lymph which was stored in contact with 50 per cent glycerine over three months at a temperature below 0°C.

The characters of eight strains of *S aureus* isolated from eight different batches of lymph soon after manufacture are given in Table IV and the characters of eight strains of the same organism isolated from the same batches of lymph after storage from three to six months are shown in Table V.

TABLE IV.

Analysis of 8 strains of S aureus.

Number	Plasma coagulation	Hæmolysis	Mannite fermentation	Toxicity
71A	+	+	+	+
76A	+	+	+	-
81A	+	+	+	+
88A	+	+	+	-
91A	+	+	+	-
93A	+	+	+	-
95A	+	+	+	-
102A	+	+	+	-

TABLE V.

Analysis of 8 strains of S aureus (after storage)

Number	Plasma coagulation	Hæmolysis	Mannite fermentation.	Toxicity
71B	—	±	—	—
76B	—	—	—	—
81B	—	±	+	—
88B	—	—	—	—
91B	—	+	—	—
93B	—	+	—	—
95B	—	—	—	—
102B	—	—	—	—

Table IV shows that all the strains were positive to the three tests, plasma coagulation, hæmolysis and mannite fermentation, and two of them, Nos 71A and 81A, were toxic to rabbit skin in addition, as seen in Table V, however, the two latter lost their toxic character and none of them gave positive plasma coagulation, only one strain retained the power to ferment mannite and four strains retained the hæmolytic property. It appears, therefore, that even though some batches of lymph may contain toxic strains of *S aureus*, they lose this character after storage in contact with glycerine.

It also appears that the strains of *S aureus* lose their toxic character more readily than hæmolytic and other properties. Two strains whose broth culture filtrates were highly toxic to rabbit skin, causing severe inflammation and necrosis, when subcultured in series on agar lost the toxic character after about 12 serial subcultures, but plasma coagulation, hæmolytic and other properties were retained even after the 40th subculture on agar.

Regarding *S albus* none of the 80 strains studied were found toxic to rabbit skin. Not one single strain showed all the other three pathogenic properties, viz plasma coagulation, hæmolysis and mannite fermentation, simultaneously.

Considering the extent of the skin reaction the toxin produces, the importance of the toxic strains of *S aureus* cannot be ignored although such strains occur only rarely when adequate precautions are taken in manufacture. It has now been adopted as one of the routine tests for the purity of calf lymph at this Institute. This procedure has not affected the manufacture in any appreciable degree as the number of batches of lymph which had to be rejected on account of the presence of toxic *S aureus* were only three out of 600 batches of lymph manufactured in four years.

SUMMARY

Details regarding a study of several strains of *staphylococci* isolated from calf lymph are given in this note. Only two of 373 strains of *S aureus* were found toxic to rabbit skin but not to monkey skin. All other strains were negative including 80 strains of *S albus* and 132 strains of *S aureus* isolated from lymph after three months' storage. It appears that the toxic property is readily lost on storage in 50 per cent glycerine but the other properties persist longer. It is suggested that batches of lymph containing strains of *S aureus* which produce exotoxin should not be issued for field use. By adopting this as a routine test, there has been no appreciable loss in the manufacture on account of rejection of lymph due to the presence of toxic *S aureus*.

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I express my thanks to Dr C G Pandit for various suggestions during the course of this work and to Lieut-Colonel H E Shortt, I M S, the Director, for permission to publish this paper.

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ACTIVITY OF 2-SULPHANILAMIDO-4-METHYLTHIAZOLE
AGAINST TYPE I PNEUMOCOCCUS INFECTIONS
IN MICE

A PRELIMINARY NOTE

BY

A N BOSE,

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AND

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[Received for publication, November 18, 1940]

FOLLOWING the introduction of sulphanilamide various derivatives of this interesting compound are being prepared and the pharmacological characteristics determined, especially to find out a drug that might be less toxic and at the same time exert a more widened activity. Most of the compounds are, however, being found either to possess no special anti-bacterial activity, or if so, extremely toxic for clinical use. By substituting the amide hydrogen of sulphanilamide by other chain or nucleus, compounds are being isolated which are of considerable therapeutic interest. In continuation of our previous work (De and Basu, 1938) we prepared certain compounds by reacting p-acetyl amino benzene sulphonyl chloride with various 2-amino thiazoles, but in the meantime several papers (Fosbinder and Walter, 1939, Lott and Bergem, 1939, McKee *et al*, 1939, Rake *et al*, 1940) have already appeared on the chemotherapy of sulphathiazole compounds. These are being found to possess a specific action against pneumococcus as well

as *Staphylococcus aureus* (Herrell and Brown, 1939) Accordingly, investigations are in progress to find out the therapeutic activity of the compounds prepared in this laboratory and the present paper deals with the protective action of 2-sulphanilamido-4-methylthiazole (I) in type I pneumococcus infections in mice

EXPERIMENTAL

2-sulphanilamido-4-methylthiazole was prepared from 2-N⁴-acetyl sulphanilamido-4-methylthiazole (Fosbinder and Walter, *loc. cit*) by refluxing the above acetyl compound with 5 per cent hydrochloric acid in dilute alcoholic solution. The hydrolysed solution was diluted with ammonia and the solid found was crystallized from dilute alcohol, when the compound separated in microscopic pale buff coloured needles, m p 236°C to 238°C. It is sparingly soluble in water, but readily dissolves in dilute acids and alkalis.

Assessment —In the experiments described below the drug has been given to mice in 2 per cent gum tragacanth suspension by means of a lachrymal cannula with a bulb-point. The animals used weighed about 22 g to 25 g. The amount of drug given at each administration and the number of animals used are stated in the respective protocols. The organism for producing the infection was a type I pneumococcus taken from a stock laboratory strain. The maximum virulence of the strain was maintained by repeated passage through mice. The organisms were then cultured in serum broth for 18 hours at pH Ca 7.8. The evaluation of the minimum lethal dose was made by determining the highest dilution of organisms that would kill 50 per cent of mice. It was usually noticed that 0.2 c.c. of a 10⁹ dilution of the 18-hour broth culture of pneumococci was the minimum lethal dose. The original 18-hour broth culture was then so diluted as to afford 1,500 m.l.d. per 0.3 c.c. which was the injecting dose. The observations were made over a period of 7 days in all cases studied.

The protective action was also noticed by subcutaneously treating the mice with the thiazole derivative in watery suspension (0.8 per cent) containing sodium bicarbonate (0.065 g per c.c.) to ensure an even distribution and consequent better absorption. The drug ($\frac{1}{2}$ c.c. of the suspension 4 mg) has been given on three occasions for both the experiments (*vide* Table II) half an hour before time of inoculation, 6 hours afterwards and then once after 24 hours. The number of mice used in each experiment was 8 and the control was made with 6 animals.

A relative therapeutic activity of the thiazole was compared with that of 2-(p-amino benzene sulphonamido) pyridine (*vide* Table III). In this case the mice were previously fed with a dose of the drug in 2 per cent gum tragacanth one day before injection. The average survival time has always been calculated by totalling the number of days survived by each mouse and then dividing by the number of animals used in each investigation according to the procedure followed by Whitby (1937).

TABLE I

*Protection by sulphamethylthiazole on mice (average weight 22 g to 25 g)
against pneumococcus type I*

Infecting dose 1,500 mld from 18-hour broth culture

Day of observation after infection	CONTROL.		GROUP A. TOTAL DOSE 50 MG			GROUP B TOTAL DOSE 75 MG		
	Dead.	Survived	Daily dose fed.	Dead	Survived	Daily dose fed.	Dead	Survived
1	0	12	20	0	12	30		12
2	12		20	0	12	30	1	11
3			10	2	10	15	3	8
4				4	6		1	7
5				2	4		1	6
6				0	4		0	6
7				2	2		0	6

TABLE II

*Mice treated subcutaneously with sulphamethylthiazole (0.8 per cent suspension)
(average weight 22 g to 25 g)*

Total dose of drug injected 12 mg

Total number of mice treated in each experiment 8

Infecting dose	Control 6 mice	Number of mice dying on a given day after infection							Survived	Average survival days (Max —7)
		1	2	3	4	5	6	7		
1,500 mld.	All dead within 24 hours	0	0	0	2	0	0	0	6	6
5,000 mld	All dead within 24 hours	0	0	0	1	0	4	0	3	5.5

TABLE III

Oral ingestion of the drug on mice (total animals 20, average weight 22 g to 25 g)

Infecting dose 1,500 mld from 18-hour broth culture

Day	Intake of drug, mg	NUMBER OF MICE SURVIVING WITH		
		Sulphamethylthiazole	Sulphapyridine	No drug
1st	20	20	20	16
Intraperitoneal inoculation with pneumococcus type I				
2nd	20	20	20	10
3rd	20	20	20	
4th	20	16	14	
5th	20	12	14	
6th		12	12	
7th		12	12	
8th		10	12	
9th		10	10	

TABLE IV

Summary of all experiments on oral administration of drug

Dose of drug, mg	Infecting dose, mld	Total number of mice used	Average survival days (Max —7)
Nil	1,500	12	1
	1,500	16	0.63
Sulphamethylthiazole			
50	1,500	12	4.2
75	1,500	12	4.7
100	1,500	20	5.1
Sulphapyridine			
100	1,500	20	5.2

DISCUSSION AND CONCLUSIONS

There is little available data concerning the experimental use of sulphamethylthiazole (Herrell and Brown, *loc cit*, Ivánovics, 1940). The present work was mainly undertaken to have an idea on the efficiency of the drug in the control of pneumococcus infections in mice. The results so far obtained indicate that this compound exerts a definite protective action whether ingested orally or subcutaneously (*vide* Tables I to III). In relative comparison with 2-(p-amino benzene sulphonamido) pyridine (sulphapyridine) (*vide* Table III), it is being noted that it is almost as active as the latter compound which is being clinically found to be efficacious against pneumococcal pneumonia. The slight difference in the average survival days, 5.1 and 5.2, as found in Table IV, may be neglected if the survival period be extended to the 8th day after infection when the mortality rate in both the groups of animals treated with the different drugs comes to the same percentage (*vide* Table III). The evaluation of this new chemotherapeutic drug necessitates an extensive experimental investigation regarding its chronic toxicity, effects on different body tissues and organs, as well as its rate of absorption and excretion. Work in this direction is already in progress. The autopsy and the blood culture of the dead animals showed pneumococcal infection, and it may be pointed out here that none of the animals so far treated with sulphamethylthiazole showed any untoward symptoms other than those of the infections. From a comparative therapeutic activity of sulphapyridine with sulphathiazole, Litchfield, White and Marshall (1940) have very recently opined that both are equally active. But the compound sulphamethylthiazole may be more easily prepared. Accordingly, its anti-pneumococcal activity demands a thorough clinical investigation in finding out a drug with a lower toxicity against this dreadful disease.

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An opportunity recently occurred of observing the effect of oral administration of some drugs during their excretion in the bile in the case of a patient with a biliary fistula following operation

EXPERIMENTS

It was found that samples of bile drained through the fistula were invariably contaminated. The samples were therefore passed through small L₃ Pasteur-Chamberland candles and the sample of filtrate obtained was inoculated into broth medium and incubated at 37°C for 48 hours. If no growth was visible during this period the sample was considered sterile.

Three organisms which are usually considered to cause infections of the biliary tract, viz (1) *B coli communis*, (2) *B typhosus* and (3) *Streptococcus haemolyticus*, were chosen for the study.

The experiments were conducted to study the following points —

- (a) Whether after administration of hexamine or sulphanilamide, bactericidal or bacteriostatic properties develop in the bile,
- (b) Whether the effect can be studied *in vitro* with hexamine.

The bacteriostatic or bactericidal properties of sulphanilamide *in vitro* have been so extensively studied that these experiments were not repeated.

(a) About 20 c c of bile was collected from the fistula and as mentioned before filtered through L₃ candle.

A normal sample of bile before administration of drug was tested for bacteriostatic properties and served as control.

The samples of bile after administration of the drug were collected for 7 days after administration of hexamine and for 5 days after administration of sulphanilamide. The doses of drug administered are recorded in Tables I and II.

The test was conducted as follows —

Six tubes were set up, the first tube was an empty sterile test-tube, the second tube contained 1 c c of nutrient broth, the others contained nutrient broth in 2 c c quantities.

Into the first tube 2 c c of the bile sample were introduced maintaining the usual aseptic precautions.

Into tubes Nos 2 to 5, graduated quantities of the bile were added to the broth, and the final volumes made up to 2.5 c c where necessary with the addition of broth. Tube No 6 contained only broth.

Three such sets were put up.

The first set was inoculated by a 2-mm diameter loop with a young culture of *B coli communis*. The second set was inoculated similarly with *B typhosus*. The third set was inoculated with *Streptococcus haemolyticus*.

All these experiments were repeated twice and gave identical results. The data are reproduced in Tables I and II.

(b) The second part of the work was undertaken because it was not possible to analyse chemically the different samples of bile for detecting the presence of hexamine or its split products, or to determine quantitatively the actual concentration of hexamine that would be necessary to confer the bacteriostatic property to bile

For this part of the test, we took 9 tubes with 2 c.c. of broth and added hexamine in graduated quantities to the first 8 tubes. The last tube did not have hexamine and served as a control.

Three sets were put up, and as in the first part of the work were inoculated respectively with *B. coli communis*, *B. typhosus* and *Streptococcus hemolyticus*.

The data are reproduced in Table III.

In the experiment, the pH of the samples of bile after administration of hexamine was also recorded with the glass electrode with the view of determining whether the inhibitory effect might be due to the alteration of the pH of the bile from its normal limits. The pH is recorded in Table I and enables us to study whether there is actually any relationship of the pH with the bacteriostatic property developing in the samples.

DISCUSSION

Hexamine (Table I)—Bacteriostatic effect of hexamine on *streptococci* was noticed after 160 grains had been administered, the growth being definitely scanty though there was hardly any effect with this dosage against *B. typhosus* and *B. coli*. This effect was gradually more and more marked and the maximum control of the growth could be seen after 480 grains of the drug, when the bacteriostatic effect was obtained even in a dilution of 1 in 25, the growth being practically negligible. Full control in all the dilutions was obtained after a dosage of 560 grains.

The growth of *B. typhosus* on the other hand was inhibited to an appreciable degree in undiluted bile only after a dosage of 240 grains. The effect reached maximum after 560 grains but even at this dose the dilute bile showed practically no effect on the *B. typhosus* (cf. *streptococci*), whereas *B. coli* after 560 grains was found to be just inhibited even in pure bile, and in diluted samples naturally the growth was fairly heavy. The bacteriostatic effect of bile after administration of hexamine against *B. coli* is minimal as compared to its effect on *streptococci* and *B. typhosus*.

As regards the pH variations and the relationship with the bacteriostatic effect after hexamine administration, it may be seen from Table I that the variation was more or less towards the alkaline side. However, the variation did not show any definite progressive change and no definite relationship between the bacteriostatic action and pH could be ascertained.

To sum up, the bacteriostatic effect of bile after administration of hexamine was highest against *streptococci*, least against *B. coli* and intermediate against *B. typhosus*.

It will be of interest to compare the above results with those of Table III which shows the effect of hexamine in different concentrations *in vitro* against three organisms. The results to a remarkable extent correspond with the findings of Table I. We find that hexamine even up to 0.3 per cent inhibits the growth of *streptococci* up to 72 hours. In the same concentration, the growth of *B. typhosus* was inhibited up to 48 hours, but there was absolutely no effect on *B. coli*. *B. coli* growth was only inhibited at a concentration of 6.20 per cent.

Clinically, there are undoubted cases of satisfactory response of gall-bladder infection with massive hexamine therapy (with alkalis). From the above observation one feels justified in stating that it is reasonable to expect control of gall-bladder infection especially due to *streptococci* with hexamine.

Sulphanilamide (Table II) —The bacteriostatic effect was comparatively more marked against *streptococci* after a dose of 28 grammes per oral administration in undiluted bile, but *B. coli* and *B. typhosus* showed a considerable growth even after this rather heavy dose. The inhibitory effect was noticed to some extent in undiluted bile against *B. typhosus* after 32 grammes, but there was no effect against *B. coli* even after this dosage. The bacteriostatic effect against *streptococci* was noticed in a dilution of 1 in 5 of bile after 32 grammes.

SUMMARY

1. A patient with biliary fistula was administered hexamine and sulphanilamide orally, to study the bacteriostatic effect of the excreted bile *in vitro* against *streptococci*, *B. typhosus* and *B. coli*.

2. The pH of the excreted bile after hexamine administration was also recorded.

3. The bacteriostatic property of the bile, after administration of hexamine as well as sulphanilamide, was most marked against *streptococci*. The effect on *B. typhosus* was comparatively less satisfactory and *B. coli* reacted the least.

4. The results with hexamine were confirmed also *in vitro*.

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TABLE I.
Showing the bacteriostatic property developing in bile after administration of Iccaxanne (orally)

[illegible]

Showing the bacteriostatic property developing in bile after administration of sulphamylamide (orally)

Bile sample numbers	Total quantity of sulphamide administered, grammes
1	NL
2	4
3	12
4	20
5	28
6	32

TABLE III

Showing the percentage of hexamine added in vitro to show bacteriostatic effect against *B. coli* communis, *B. typhosus* and *Streptococcus haemolyticus*

Tube numbers	Broth, c c	Hexamine, gr	Per cent	<i>B. coli</i> communis			<i>B. typhosus</i>			<i>Streptococcus haemolyticus</i>		
				24 hours	48 hours	72 hours	24 hours	48 hours	72 hours	24 hours	48 hours	72 hours
1	2	$\frac{1}{4}$	25	—	—	—	—	—	—	—	—	—
2	2	$\frac{3}{8}$	18.75	—	—	—	—	—	—	—	—	—
3	2	$\frac{1}{2}$	12.5	—	—	—	—	—	—	—	—	—
4	2	$\frac{1}{8}$	0.25	—	—	—	—	—	—	—	—	—
5	2	$\frac{1}{20}$	2.5	+	+	+	—	—	—	—	—	—
6	2	$\frac{1}{10}$	1.25	+	+	+	—	—	—	—	—	—
7	2	$\frac{1}{80}$	0.025	+	+	+	—	—	±	—	—	—
8	2	$\frac{1}{160}$	0.012	+	+	+	—	—	±	—	—	—
9	2	Nil (control)		+	+	+	+	+	+	+	+	+

Note—In all the tables the growth of organisms is represented as —

+ + + + = Heavy
 + + + = Fair
 + = Scanty
 ± = Very scanty
 — = No
 — = Steady

BALANCED DIETS

Part II

STUDIES ON THE NUTRITIVE VALUE OF FISH.

BY

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IN the formulation of cheap and well-balanced dietaries difficulty is often encountered in finding foodstuffs which are rich in proteins of high biological value and at the same time cheap. Although milk, meat and eggs are the chief sources of proteins of high biological value they do not form any appreciable part of the daily diet of poor people due to their high cost. A survey of dietary habits of poor non-vegetarian groups of people in Bombay revealed that they consume a large quantity of fresh fish in preference to meat. In the still poorer stratum of the population salted and dried fish is taken probably because of its comparative cheapness.

In Europe as well as in America a large volume of work has been done on the nutritive value of fish (Atwater, 1887, Williams, 1911, Clark and Almy, 1918, Dill, 1921, Shostrom, Clough and Clark, 1924, Gibbs, 1927, Peterson and Elvehjem, 1928). In India some investigations have been carried out by Guha and his colleagues on fresh-water fish in Bengal (Chakravorty, Mookerjee and Guha, 1933, Ghosh, Chakravorty and Guha, 1933, Ghosh and Guha, 1934, 1935, see also Basu, Sircar and Gupta, 1940) and by De, Majumdar and Sundararajan (1938) and Seshan (1940) in Madras. The former group of investigators has shown that Bengal fresh-water fish provides a rich source of proteins of high biological value

and minerals, such as calcium, phosphorus and iron. They further found that the liver oils of some of the varieties of fish investigated, were considerably richer in vitamin A than an average sample of cod-liver oil, the body fats, however, were found to be devoid of vitamin A.

A study of the literature has shown that no systematic investigation has been made of the nutritive value of fish caught in coastal waters of Bombay and the Konkan and consumed by the inhabitants as fresh or as salted and dried fish.

EXPERIMENTAL

Selection of the sample—The procedure adopted in sampling depended upon the size of the fish under examination. If the fish was very small a number sufficient to weigh about 500 g. was taken in one lot. In the case of a big-sized fish a portion of about eight inches square and three inches deep was cut out from the back near the middle of the body. The edible portion in either case was separated. The fleshy portion was minced and the pulp subjected to analysis as follows—

Analysis—The minced edible portion of the fish, separated from the bones and other non-edible portions as stated above, was dried at 45°C. in a current of hot air and powdered. When dried fish was being analysed this preliminary drying was not necessary.

The moisture content was determined on a fresh weighed sample of fish by drying it to constant weight in a steam-oven at 100°C. The estimation of nitrogen was carried out by Kjeldahl's method, the amount of N thus obtained was multiplied by the factor 6.25 for obtaining the total proteins. The amount of fat was determined by extraction of the dried material with ether. The ash was estimated by Stolte's method described by Peters and van Slyke (1932).

The ash was dissolved in dilute hydrochloric acid, filtered from insoluble residue and washed with water. The filtrates and washings were made to a known volume and aliquots of this solution were used for the determination of phosphorus, calcium and iron. Phosphorus was estimated by Brigg's (1922) modification of the Bell and Doisy method, calcium by the volumetric permanganate method described by McCrudden (1909, 1911) and iron according to Kennedy (1927). In Tables I and II are given the results of analyses of dried and fresh fish respectively. These results are the means of two samples collected at different times, the duplicate samples of each variety collected at different times did not vary beyond 2 to 3 per cent in composition. The ash content of the dry fish has not been included in Table I as the ash in this case would contain a large proportion of NaCl due to the common salt used in the process of salting and drying.

ESTIMATION OF VITAMIN A

The method of Carr and Price (1926) as modified by Coward *et al* (1931) was adopted for the investigation of the vitamin A content of fish-liver oils.

The finely minced liver was ground up with anhydrous sodium sulphate and extracted repeatedly with ether in the cold, the ethereal extract was distilled in the

TABLE II
Analysis of fresh fish

Local name	Scientific name	Edible portion, per cent	CONTENTS IN 100 G OF THE EDIBLE PORTION							
			Moisture, g	Protein, g	Fat, g	Calcium, mg	Phosphorus, mg	Iron, mg	Ash, g	Insoluble inorganic matter, g
1 Kajura	<i>Lates calcarifer</i>	93.30	79.40	12.58	0.892	54.08	89.29	1.156	0.0160	0.917
2 Surmai	<i>Cyprum kuhli</i>	87.20	63.00	19.86	1.370	92.54	161.75	2.013	0.0305	3.300
3 Ghol	<i>Scæna miles</i>	91.70	69.70	18.39	0.898	88.57	153.20	2.059	0.0233	2.370
4 Singhada	<i>Arius dussumieri</i>	87.00	61.00	20.87	3.091	97.50	161.71	1.783	0.0322	2.124
5 Rangoli		86.70	66.60	16.86	1.206	73.00	113.28	1.905	0.0237	1.625

TABLE III

Vitamin A in fresh-liver oil

* Average cod-liver oil 118 blue units per gramme of oil

Local name	Scientific name	AVERAGE LENGTH OF THE FISH IN INCHES		APPROXIMATE BREADTH OF THE FISH IN INCHES*		WEIGHT OF LIVER IN GRAMMES		PERCENTAGE OF OIL		Vitamin A blue units per gramme of oil
		Maximum	Minimum	Maximum	Minimum	Maximum	Minimum	Maximum	Minimum	
1 Hala	<i>Strumileus niger</i>	12	12	12	11	160	67	2.09	2.18	5,177
2 Karh	<i>Chirocentrus</i>	18	14	6	6	100	81	12.10	11.08	5,104
3 Kojura (bogu)	<i>Lates calcarifer</i>	24	18	8	6	210	64	9.37	9.04	8,374
4 Ravas (small)	<i>Polygynus tetractylus</i>	21	18	8	4	60	56	3.10	1.27	99,265
5 Ravas (big)	<i>Sciaenops blockeri</i>	40	24	11	6	231	48	15.30	12.01	9,494
6 Mushu	<i>Scorpaenopsis scorpaenoides</i>	60	40	18	14	1,110	230	52.5	47.50	6,780
7 Singbadu	<i>Arctus tussimieri</i>	18	13	6	4.5	210	68	18.15	13.60	178
8 Toki (yakti)	<i>Trichurus savala</i>	24	17	4	3.5	55	12	8.4	6.30	839
9 Surmai	<i>Cybinus lulu</i>	12	9.5	6	5.3	120	60	3.15	3.05	1,118
10 Shongti	<i>Macropodus chinensis</i>	11.0	12.0	12	9.0	120	100	30.00	28.04	6,403
11 Rangoli		24	12	8	3	183	48	8.62	6.81	15,570
12 Mandoli	<i>Coilia dussumieri</i>	12	12	6	4	108	102.6	3.60	3.38	20,007
13 Dhumi		18	12	6	3	60	43	6.95	6.54	1,717
14 Ghol	<i>Sciaenops niger</i>	28	24	12	11	230	130	17.70	14.02	6,532
15 Wagh	<i>Dasyatis imbricata</i>	90	88	36	35	620	570	21.50	21.20	538

* The figure of 118 blue units per gramme for the average cod liver oil was arrived at by finding the mean of a number of observations on several samples of the oil supplied in bulk to the K. E. M. Hospital, Bombay.

presence of nitrogen and the solvent completely removed. Traces of moisture were removed by drying the oil in vacuum over P_2O_5 . A series of dilutions of oil were prepared in dry redistilled chloroform. The intensity of the colour produced when 0.2 c.c. of each diluted solution was mixed with 2 c.c. of antimony trichloride reagent in a cell, was matched with Lovibond tinted glass (the colour being matched within 30 seconds). Six or more readings were taken in each case and their mean found. The results of vitamin A content of liver oils expressed in terms of blue units as read on the tintometer and calculated per g. of oil are given in Table III.

DISCUSSION

In Tables I and II are given the results of the analysis of thirteen varieties of the dried fish and five varieties of fresh fish respectively. As was expected, all of them contain large amounts of proteins.

Schormuller (1937) working on the digestibility of the proteins of fish with pepsin and trypsin has come to the conclusion that fish proteins are easily digestible and the digestibility is comparable with that of egg albumin. Basu and Gupta (1939) working on the biological value of the proteins of some species of Bengal fish by the nitrogen balance and the growth method have concluded that the different fishes studied by them contained a large percentage of proteins of very good quality.

It has been mentioned that fish is comparatively a cheaper article of diet than meat, milk or eggs. The price of salt water fish varies with the type of fish and the amount of the catch. Although most varieties of freshly caught fish are cheaper

mutton, there are a few which are considerably more expensive. There are, however, mostly consumed by poorer people which are even cheaper than

of

The salted and dry fish works out cheaper than fresh fish as regards protein content even though their cost per pound of material be the same. Thus 100 g. of fresh fish yield between 12.58 g. and 20.87 g. of protein whereas the same quantity of fish will yield from 27.77 g. to 76.07 g. of proteins. The same can be said of the mineral contents.

VITAMIN A CONTENT OF LIVER AND BODY OILS

In Table III are given the values of vitamin A content of fifteen common varieties of fresh fish-liver oils. In the table are also included the average weights and the percentages of oils of fresh fish livers studied. The majority of these oils were found to be rich in vitamin A, its amount being several times larger than that found in an average sample of cod-liver oil. These results open up a new possibility of utilizing local sources of supply of oil rich in vitamin A for therapeutic purposes. Further work on the seasonal variation of the yields of oil and of vitamin A from these fish livers is in progress.

An inquiry into the food habits of fish-eating class of people has shown that fish livers are not normally eaten. In view of their rich vitamin A content it would

seem desirable to encourage the practice of eating fish liver, though the question of its palatability is an important factor to be considered

The chemical tests did not reveal the presence of vitamin A in the body fats of these varieties of fish

SUMMARY

1 Thirteen varieties of dry and five varieties of fresh sea fish have been analysed for proteins, fats, calcium, phosphorus and iron. The results show that these fish constitute a cheap source of animal proteins and essential minerals such as Ca, P and Fe

2 The liver oils of some of these fish, namely rivas, ghol, shengti, mushi, and halva were found to be several times richer in vitamin A content than ordinary cod-liver oil. The body fats of dried and fresh fish do not probably contain vitamin A in appreciable amounts

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higher during the pre-menstrual period and lower during the menstruation (Wakeham, 1923) For the sake of uniformity in the comparison of data the tests were done in the non-menstrual period between the fourth and tenth day after menstruation

Apparatus and technique—These were the same as reported in our first paper (Niyogi *et al*, 1939) The closed circuit type of apparatus was used for determining the oxygen consumption and the mean of the second and third day series of observations which was invariably lower than the average of first day readings, was taken as the true figure for calculating the result The heat production was calculated by reference to the tables on the assumption that the R Q in the post absorptive stage was 0.82

Estimation of creatinine—This estimation is based on the red colour which develops when an alkaline solution of picric acid is added to a solution of creatinine This is compared with the colour of a standard creatinine solution The estimation was carried out according to the method described by Peters and van Slyke (1932)

Creatinine coefficient—This was calculated by dividing the number of milligrams of creatinine excreted daily by the body-weight measured in kilograms

Total urinary nitrogen—This was estimated by Kjeldahl's method

THE NATURE AND PURPOSE OF INVESTIGATION

The basal metabolism is subject to a certain margin of variation due to the operation of a number of factors which so far have proved incapable of control Among these may be mentioned variation due to technique, changes in the nervous tension of the subjects and the degree of muscular relaxation attainable DuBois (1936) mentions that an error of 1 to 2 per cent is likely to occur in case of close-circuit determinations in which a respiratory quotient of 0.82 is employed for the calculations Too much reliance, however, should not be placed upon the value of the respiratory quotient as an unequivocal criterion of the oxidative metabolic activity The respiratory quotient in a given case is undoubtedly the resultant of several distinct metabolic processes—synthesis, interconversions as well as combustion The constancy of metabolism under a great variety of conditions may not represent so much a constant state of cellular activity but may rather reflect a lack of refinement in the technique

The metabolic rates determined on the lowest readings of successive determinations are about 3 per cent lower than the rates calculated on the averages of the first day readings Even when due allowance is made for the metabolic rate being influenced by these causes there still remains a large difference to be accounted for and we must look to some other factors to explain the low B M R of the subjects that we have investigated The low metabolism has been attributed to one or more of several factors, such as low protein intake, low blood pressure, excessive humidity and high temperature of the atmospheric air, undernutrition, greater degree of physical and mental repose noticeable in people living in the tropical and

sub-tropical regions and to some inherent racial peculiarity. The data incorporated in the present communication have been utilized for the discussion of the relative importance of the above factors in the lowering of metabolism.

INFLUENCE OF DIET

Benedict and Roth (1915) did not find any significant differences in the metabolism of the vegetarians and non-vegetarians and this was confirmed by Harris and Benedict (1919). McClellan, Spencer and Falk (1931) investigated the basal metabolism of two Arctic explorers living for two months on an exclusively meat diet in the Sage calorimeter, but they did not notice any significant change in metabolism. Krogh and Lindhard (1920) on the other hand found a decrease of metabolism on a low protein diet. Bierring (1931) confirmed this finding in one trained subject. Deuel (Deuel *et al.* 1928) also has reported a drop in his own metabolism when he was on a non-protein diet. In view of the contradictory results obtained by different workers experiments were undertaken to find out if there existed any relationship between basal metabolism and the endogenous as well as the exogenous protein metabolism and if it was possible to find in this an explanation of the low basal metabolism of Indians. As early as 1914 Palmer and his collaborator (1914) pointed out the possibility of using urinary creatinine as a basis for evaluation of basal metabolism. In a study conducted on 31 men and 21 women, Takahira (1927) found that the urinary creatinine was proportional not only to body-weight but to basal metabolism as well. Wang (1939), however, suggests that some other factor or factors aside from active protoplasmic tissue may be responsible for the production of either basal heat or creatinine or of both, as the metabolic rate and the preformed creatinine content did not run parallel at all the ages in the group of children investigated by her. It is quite conceivable that the metabolic rate does not vary so much with the level of creatinine elimination as with the level of total endogenous nitrogen elimination in the urine.

In our experiments several 24-hour samples of urine of ten healthy adult males who were members of the staff of the Physiology Department were collected and their creatinine content determined. The results are given in Table I. It will be seen that the average individual daily creatinine elimination was from 1.0 g to 1.7 g and the creatinine coefficients of the ten subjects were found to vary between 19 and 27. From the available data it appears that in Americans the daily output of creatinine amounts to 1.5 g to 2 g for men and the creatinine coefficient varies from 20 to 26. The endogenous protein metabolism of the above subjects as indicated by the creatinine coefficient does not differ materially from that of Americans. Some of the subjects having a low creatinine coefficient show a higher B.M.R. than that of individuals having a higher coefficient. The low basal metabolism of these ten persons cannot therefore be attributed to a low level of endogenous protein metabolism. With a view to ascertain whether exogenous protein metabolism has any appreciable effect on basal metabolism the same ten subjects who fully co-operated in the investigation were kept under observation. Their average daily output of urinary nitrogen and the B.M.R. were determined. Their protein intake which

TABLE I.

B M R, urinary creatinine and total nitrogen output before and after high protein diet

Subject number	Creatinine 24 hours, mg	Creatinine coefficient	Total urinary nitrogen, g	Average B M R before experiment, calories per sq m per hour	Protein intake in usual daily diet, g	Amount of extra protein fed per day, g	Total urinary nitrogen after feeding extra protein, g	B M R during high protein diet, calories per sq m per hour
						Week		
26	1,118	22.0	6.30	29.1	88.6	1st	6.87	31.6
						2nd	6.4	32.3
						3rd	6.69	30.8
27	1,068	21.4	5.99	32.9	72.5	1st	7.02	33.6
						2nd	10.11	33.2
						3rd	9.69	31.7
						4th	8.63	32.1
28	1,475	23.3	7.25	33.4	57.6	1st	9.30	32.2
29	1,572	27.1	9.30	32.0	66.2	1st	8.01	32.2
						2nd	8.47	32.2
30	1,737	26.7	9.53	32.4	82.6	1st	12.82	32.0
						2nd	14.99	31.4
						3rd	15.05	32.1
						4th	13.31	32.5

J, ME	31	1,003	10 0	7 15	11 7	70 70	1st 2nd 3rd 4th	42 42 12 42	0 21 8 07 9 50 8 28	37 1 33 0 35 2 31 4 35 3 37 5 36 2 35 5 32 0 33 3 33 1 31 2 31 9 31 5 33 2 34 5 30 7 34 1 32 7 32 7
12	12	1 016	10 1	1 87	15 5	73 7	1st	10	0 57	36 3
							2nd	10	0 31	37 5
							3rd	40	0 07	36 2
							4th	10	7 05	35 5
33	33	1,130	21 1	0 00	33 0	64 3	1st	12	7 97	32 0
							2nd	12	8 10	33 3
							3rd	12	0 52	33 1
							4th	42	8 40	31 2
34	34	1,017	23 3	0 03	11 5	70 9	1st	10	0 5	31 9
							2nd	40	8 0	31 5
							3rd	10	8 2	33 2
							4th	40	7 8	34 5
35	35	1,055	25 7	5 56	35 0	63 4	1st	40	7 0	30 7
							2nd	10	8 8	34 1
							3rd	10	7 3	32 7
							4th	40	8 9	32 7
CO AVERAGES		1,210	23 2	0 05	31 07	72	1st	30	8 51	33 5
							2nd	13	8 00	33 2
							3rd	40	0 11	33 1
							4th	43	8 00	33 2

was found to be 72 g on an average was increased by the daily addition of meat, fish or eggs equivalent to a supplement of 40 g to 60 g of protein per day for nearly a month. Increase in the daily nitrogen output followed. Weekly observations of the B M R and the estimation of urinary nitrogen were made during the period the subjects were on extra protein diet. The results are included in Table I. It will be seen that the average daily output of nitrogen is only 6.95 g in the pre-experimental period, a figure which is much below the European average. The low urinary nitrogen output in Indians has also been recorded by Sokhey and Malandkar (1939). After the intake of extra protein there is an appreciable increase in the nitrogen output without any significant rise of basal metabolism. It may be argued that the above experiments were of short duration and even after the administration of extra protein neither the excretion of urinary nitrogen nor the B M R reached the usual European averages. Europeans habitually consume and metabolize larger quantities of proteins and in them a large protein meal may exert, according to Sokhey and Malandkar, a specific dynamic action for more than 13 to 14 hours which represent the usual interval that is allowed to pass between an evening meal and the determination of basal metabolism done in the following morning. Even a prolonged intake of a fairly large quantity of animal protein in the diet may not necessarily raise the metabolic rate as is shown by the results reported below. We (Niyogi and Sukhatankar, 1939) had opportunities to survey the non-vegetarian and vegetarian diets of the nurses in the messes attached to the K. E. M. Hospital, Bombay. The nurses get a scheduled diet throughout the year. The vegetarian Hindu nurses consume 101 g of protein daily of which 32 g are of animal origin. The non-vegetarian Indian Christian nurses take 118 g of protein of which 80 g are derived from animal sources. The average B M R of these two groups of nurses are as follows —

- (1) Vegetarians 12 Hindu women = 33.0 calories per sq. m. per hour
- (2) Non-vegetarians 11 Indian Christian women = 31.4 calories per sq. m. per hour

It will appear that the Indian Christians who habitually consume large quantities of animal proteins have on an average a slightly larger metabolic rate than that of the vegetarians. This difference, however, is statistically not significant. It seems probable, therefore, that exogenous protein metabolism, even when maintained at a fairly high level for long periods, does not necessarily increase the metabolic rate.

INFLUENCE OF TEMPERATURE AND HUMIDITY

In ordinary metabolism studies the usual procedure is to keep the subject comfortable so that he feels neither excessive degrees of heat nor cold. A sensation of chilliness is accompanied by an increase in metabolism and shivering may increase the heat production two or four times above the basal level (DuBois, *loc. cit.*) McConnel, Yagloglou and Fulton (1924) exposed a number of men to various levels of the 'effective temperature', an index which takes care of external

temperature, humidity and air movement. They found the metabolism approximately level between 62°F and 87°F. In Bombay the average annual range of variation of external temperature is from 80°F to 86°F and of humidity is from 54 to 86 per cent. The summer lasts from March to May. The temperature and relative humidity are higher during this period than in any other season. From June to September there is heavy rainfall and the temperature and the humidity are slightly lower than in summer. October is generally warmer than the summer months although the average humidity is lower. The winter extends from November to February, the temperature and humidity being lowest during this season. The average B M R of 15 nurses during the different seasons of the year together with the atmospheric temperature and humidity are given in Table II and plotted in the Graph. The data recorded in the table clearly show that the heat production is slightly higher during the summer months. Similar findings have recently been reported by Rahman (1939). Ahmad, Lal and Roy (1938) exposed a few individuals to sudden changes of atmospheric conditions by transferring them from open air where the temperature varied from 85°F to 96°F and relative humidity between 65 and 80 per cent to air-conditioned rooms having a temperature range of 75°F to 80°F and humidity varying from 51 to 59 per cent. The metabolism was found to be higher in the hot humid atmosphere than in the air-conditioned room.

TABLE II

Temperature, humidity and B M R

Month	Average temperature, °F	Average humidity, per cent	B M R in calories per sq m per hour (average for 15 women)
<i>Summer</i>			
March	83.3	86.1	34.8
April	85.2	90.3	33.3
May	88.5	81.3	31.5
AVERAGE	85.7	85.9	33.2

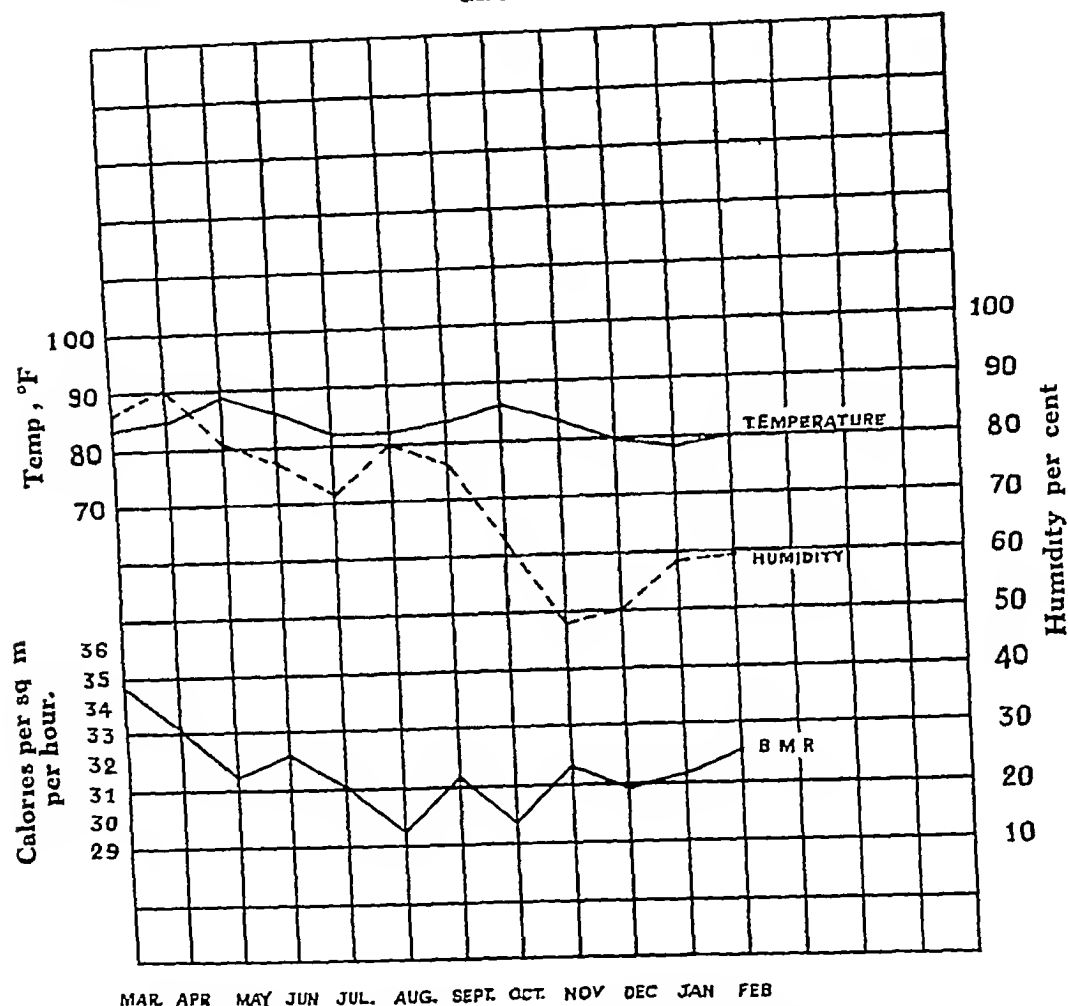
*Basal Metabolism in Bombay.*TABLE II—*concl.*

Month	Average temperature, °F	Average humidity, per cent	B M R in calories per sq m per hour (average for 15 women)
<i>Monsoon</i>			
June	86.8	77.0	32.2
July	82.3	71.5	31.1
August	82.04	80.04	29.6
September	83.5	76.7	31.4
AVERAGE	83.7	76.3	31.07
October	86.0	62.2	29.8
<i>Winter</i>			
November	83.6	48.2	31.7
December	79.5	50.0	30.9
January	78.1	58.4	31.3
February	79.3	59.4	32.2
AVERAGE	80.1	54.0	31.5

Banerji (1931) on the other hand is of opinion that high atmospheric temperature and humidity are the most important factors which lower the B M R in the Indians. According to him the B M R curve follows more closely the atmospheric humidity curve than the atmospheric temperature curve. We could not however trace any such relationship in the Graph. The B M R of our subjects is fairly uniform during monsoon and winter and there is only a slight fall in October when the temperature reaches its maximum. As the temperature, humidity and B M R curves do not show any definite relationship to one another throughout

the year it seems that the metabolism does not fluctuate much within the temperature and humidity range to which an individual is normally subjected and apparently adapted

GRAPH



The monthly variations of temperature, humidity and the average B M R of 15 female subjects

INFLUENCE OF BLOOD PRESSURE

The blood pressure (basal) determinations made in this laboratory and elsewhere in India have given readings lower than those that are considered normal for average Westerners and it is necessary to investigate the influence if any of low

blood pressure on metabolism The Chinese in general have been found to have a low blood pressure and a low metabolic rate A group of 12 men investigated by Benedict, Roth and Smith (1919) showed a low blood pressure and a low metabolism as a result of underfeeding The blood pressure and the B M R of 32 males (divided according to their level of blood pressure into groups of 10, 17 and 5) and 52 females (divided into groups of 27, 21 and 4) are given in Table III It will be seen that in the case of males the B M R shows a slight increase with rise of blood pressure but a parallel increase is not noticeable in the case of females A group of female subjects (group I) with low blood pressure shows a higher B M R as compared to that of two other groups (groups II and III) that have a higher blood pressure

Crile and Quiring (1939) carried out metabolism tests in north-eastern Canada on six male and seven female Chippewa Indians The average metabolic rates in the males and females were respectively 18 and 18.5 per cent higher as compared with the Mayo Clinic standards established for the American white population, although the blood pressure in both sexes was lower than that for the white individuals The above findings tend to show that the basal metabolic rates significantly higher than the standard estimates may be associated with a low blood pressure and this factor may not bear any relationship to the average low B M R noticed in our subjects

TABLE III

Blood pressure, vital capacity, Peldisi and B M R

—		Average age in years	Number of subjects	—	Average B M R in calories per sq m per hour
				<i>Blood pressure, systolic</i>	
<i>Males—</i>					
Group	I	26	10	96.5	33.2
	II	24/3	17	105	34.5
	III	23/10	3	118.6	35.2
<i>Females—</i>					
Group	I	21/6	27	97	32.3
	II	22/6	21	103.7	31.6
	III	21/11	4	116.7	32.0

TABLE III—concl'd -

		Average age in years	Number of subjects		Average B. M. R. in calories per sq m per hour
				Vital capacity, c c	
<i>Males—</i>					
Group	I	21/1	7	2,057	36.2
	II	24/11	11	2,472	34.2
	III	24/7	10	3,120	33.3
	IV	29/8	4	3,800	33.1
<i>Females—</i>					
Group	I	22/1	19	1,368	32.6
	II	21/6	21	1,719	31.6
	III	22/9	9	2,188	31.1
	IV	21/3	2	2,700	32.3
				<i>Pelidisi</i>	
<i>Males—</i>					
Group	I	23/8	12	89.2	34.3
	II	26/5	11	94.1	33.7
	III	24/2	7	97.9	34.6
	IV	21/9	2	102.7	35.2
<i>Females—</i>					
Group	I	23/7	6	88.5	32.9
	II	22/11	26	92.8	31.7
	III	23/2	16	98.3	32.4
	IV	22/8	4	103.3	31.0

VITAL CAPACITY IN RELATION TO BASAL METABOLISM

People of athletic and muscular build have generally a high vital capacity and it is interesting to investigate the relationship, if any, between vital capacity and the

B M R Benedict and Carpenter (1910) made certain comparisons of the basal metabolism of four athletes with that of a number of non-athletes having approximately the same body-weight and height. All four athletes showed a distinctly lower metabolism than the non-athletes. Benedict and Smith (1915) did not, however, find a characteristic lower metabolism in the athletes and a large proportion of them did show a considerable increase of metabolism, which could not be attributed to the after-effects of muscular activity on the preceding day as previously suggested by Benedict and Cathcart (1913). No opportunity presented itself to us for comparing the basal metabolism of professional athletes and non-athletes but among the subjects that we investigated there were some active young men who participated regularly in sports. The average vital capacities and B M R of several groups of our subjects are given in Table III. An examination of the data shows an apparent fall of B M R of both males and females with increase of vital capacity except in one group of females (group V) in whom an increase of vital capacity is associated with a rise of B M R. These variations, however, are not significant. These findings are, however, in agreement with those of Krishnan and Vareed (1932) who reported that the B M R of a few of their subjects that indulged in good exercise were practically the same as or below the predicted calculated values for the ages. As the B M R shows an apparent tendency to fall with athletic training and consequent increase of vital capacity, the low B M R of our subjects cannot possibly be due to a poor muscular build or a general lack of strenuous muscular activity.

INFLUENCE OF THE STATE OF NUTRITION

The state of nutrition is supposed to have some influence on metabolism. Various somatometric indices have from time to time been formulated to serve as a basis for the assessment of the state of nutrition. All these indices have been subjected to much criticism either because they require trained observers to make the necessary measurements or because the indices in each case require to be carefully interpreted. A good deal of investigation is required to be done before a suitable formula can be devised for assessing the nutritional state. In using the indices of nutrition the index of the individual is compared with the average or ideal. According to Pirquet (1922) of Vienna the Peldisi as represented by $3 \sqrt{\frac{10 \times \text{wt in grammes}}{\text{sitting height in cm}}} \times 100$ is the best possible index of the state of nutrition. With the Westerners a Peldisi of 97 or 98 represents normal nutrition, a Peldisi below 90 represents undernutrition and a value over 100 represents overnutrition and obesity. It is difficult to say whether Peldisi can be taken as a real index of the state of nutrition, for the index is such that a slight error in the measurement of height greatly affects the results. The average Peldisi and the B M R of several groups of male and female subjects are given in Table III. Although in the case of the majority of groups of male and female subjects the metabolism shows a tendency to vary inversely with the Peldisi, the association of these two could not be statistically proved. Talbot (1921) has shown that undernourished infants have a metabolism much above the average for normal children both

according to weight and surface area Wang and her associates (1925) noted a moderate increase in the metabolism of undernourished children 4 to 13 years old. It is quite likely as has been suggested by Talbot that the increase of metabolism in the above cases is due to a loss of inactive fat and a relative increase of active protoplasm per unit of body mass. Wilson and Roy (1938) have suggested the possibility of undernutrition and low protein diet being the cause of low metabolic rate of children investigated at Calcutta. The foregoing results as well as those already published (Niyogi *et al.*, 1940) do not bear out this suggestion. Since the majority of the subjects have a Peldisi above 90 undernutrition cannot be the causative factor of low metabolism of Indians.

INFLUENCE OF MUSCULAR RELAXATION

It has been stated that the low B M R of the Indians might be due to a greater degree of muscular relaxation presumed to exist in people in tropical and sub-tropical regions. The observations of Necheles (1930, 1932) go to show that an average Chinese is more relaxed than an average Westerner. In the Chinese the B M R shows very little difference when awake or asleep, whereas in the Westerners there is a drop of 10 per cent during sleep. Mason and Benedict (1934) consider that the above investigation carried out by Necheles was not done under ideal conditions which are essential in a study of the factor of sleep. They found that the degree of muscular relaxation during sleep in South Indian women was of the same magnitude as in the case of Western women and all individuals, regardless of race showed a decrease of metabolism to the extent of 10 per cent during sleep. The low B M R of Indians cannot therefore be ascribed to a greater degree of muscular relaxation during waking hours. Krishnan and Vareed (*loc cit*) suggested that the hot tropical climate is partly responsible for the muscular relaxation and lowering of basal metabolism, of the subjects which they investigated at Madras. The data presented in this paper show that the average B M R of our subjects during April, May and June when the atmosphere is more warm and humid is higher than in winter months when the days are comparatively cooler and drier. The results recorded at Hyderabad by Rahman (*loc cit*) also indicate that the B M R is slightly higher in summer (May and June) than in winter (November and December). This author attributes the high metabolism to the effects of sunlight and dryness of atmosphere. The fact that the B M R of our subjects was higher in summer in spite of the atmosphere being humid suggests that humidity may not have an important rôle in lowering the metabolism.

INFLUENCE OF RACIAL CHARACTERS

The question whether racial peculiarity is a specific factor in influencing the metabolism has been very extensively studied. Macleod, Crofts and Benedict (1925) investigated the basal metabolism of Chinese and Japanese women in the Physiological Laboratories of Mount Holyoke College and Teachers' College, New York, and found their basal metabolism to be distinctly lower than the standards

for American women of like age, weight and height, although the oriental students were living under the same conditions of temperature and humidity as their American College mates, engaged in the same degree of athletic exercise, all partook of the same food in essentially the same normal amounts and there was nothing abnormal in their physical configuration and development. In an archaeological expedition to Chichen Itza, Yucatan, the basal metabolism of white men and women and Maya Indians was determined by Williams and Benedict (1928). The place lies 20 degrees north of the equator, the climate is sub-tropical and the shade temperature varies from 80°F to 85°F. The Mayas represent the descendants (both mixed with Spanish and relatively unmixed) of one of the primitive American races. They are short in stature but on the whole they are as muscular as white men. The B M R of the white members of the expedition did not show any significant variation during and following a short stay at Yucatan. A group of 32 Mayas was found to have a metabolism averaging 5.2 per cent above the standards for white men and there were reasons for believing that the high metabolism was due to racial characteristics rather than to dietetic habits, living conditions or differences in bodily configuration. Mason (1934) working at Madras on 34 European and 54 Indian women found their B M R to be respectively 8 and 17 per cent below the Benedict standards. The above difference of 9 per cent could only be attributed to factors other than climate. Rajagopal's (1938) investigations at Coonoor at an altitude of 6,000 feet above the sea-level showed the average basal metabolism of 26 Indians to be 8 per cent below the average for 20 European subjects and this difference was believed to be due to racial factor. Our results indirectly point to the racial factor being the main cause of the low basal metabolism of Indian subjects as we found that diet, climate, low blood pressure, undernutrition and other factors which are generally believed to lower the basal metabolism had no such influence on it.

CONCLUSIONS

1. A high protein intake and an increased nitrogen elimination are not accompanied by an increase of basal metabolism.

No significant differences were observed in the B M R of strictly vegetarians and that of non-vegetarians.

2. The average creatinine coefficient of adult males in Bombay is practically the same as that of the Westerners and yet their basal metabolism is low.

3. Individuals with low creatinine coefficient do not show a low metabolism.

4. The level of basal metabolism seems to be uninfluenced by the normal variations in blood pressure, vital capacity and Pelidisi.

5. In Bombay the difference of temperature and humidity of the atmospheric air is not very great during the different seasons and although no marked change of metabolism has been noticed during the year there appears to be slight increase of metabolism in the summer months when the atmosphere is hot and humid.

6 The fact that dietetic, climatic and other factors do not lower the basal metabolism is a strong though indirect evidence in favour of the racial characteristic being the cause of low metabolism in our subjects

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ELIMINATION OF EXCESS NERVE TISSUE FROM ANTIRABIC VACCINE

BY

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INTRODUCTION

ANTIRABIC VACCINES at present in use are, for the most part, crude suspensions of brain from sheep or rabbits in the late stages of infection with fixed rabies virus. The vaccine commonly used in India is a 5 per cent suspension of fixed virus sheep-brain in carbol-saline prepared according to the method introduced by Semple (1911). This and other antirabic vaccines contain a large amount of nerve tissue substance which is unlikely of itself to have any antigenic value and the injection of which is aesthetically undesirable and may be harmful. Antirabic vaccines are often administered in large doses over relatively long periods of time thereby causing considerable inconvenience to the patient by loss of time and by the production of irritating local (and occasionally general) reactions. It would obviously be desirable to prepare a vaccine which would remove some of these disadvantages. As a first step in this direction, the possibility of eliminating some of the nerve tissue present in antirabic vaccine without impairing its antigenic value was investigated.

MATERIALS AND METHODS

Preliminary experiments showed that it was possible to eliminate a considerable proportion of nerve tissue from sheep-brain suspensions by precipitation at the isoelectric point. Suspensions of fixed virus sheep-brain were prepared in buffer solutions adjusted to pH 4.4. The most suitable buffer for this purpose which has so far been investigated was the glycine-acetate-phosphate system of Northrop and de Kruij (1922). When tested, the clear supernatant resulting from precipitation by this method appeared to contain a high concentration of rabies virus, though this was difficult to estimate accurately since the virus was quickly rendered non-infective to animals by exposure to greatly lowered pH.

The clear supernatant obtained after precipitation by the method described was rendered non-infective by incubation at 37°C for 24 hours at the pH obtained, neutralized by titration with N/10 sodium carbonate solution, and subjected to test for its immunizing properties

The clear supernatant (clear vaccine) used in the experiments to be described was obtained by precipitation of 10 or 20 per cent suspensions of fixed virus sheep-brain in buffer solution. Estimations of the protein content of various preparations showed that the total protein present in the clear supernatant from 20 per cent suspensions was approximately equal to that present in whole 5 per cent vaccine prepared by Semple's method (phenol vaccine). The protein content of the supernatant from 10 per cent suspensions was approximately half that of ordinary 5 per cent phenol vaccine. An example of actual estimations is given in Table I —

TABLE I

Homogeneous suspension of sheep brain	Total protein content of whole suspension in grammes per 100 c c	Total protein content of clear supernatant after precipitation in grammes per 100 c c
5 per cent (phenol vaccine)	0.437	
10 per cent suspension	0.956	0.188
20 per cent suspension	1.844	0.385

In the experiments to be described the protein content of the clear vaccine was adjusted to be equal to, or half, that of 5 per cent phenol vaccine.

The immunizing properties of clear vaccine were investigated in guinea-pigs, in parallel with 5 per cent phenol vaccine. In some experiments, the precipitate remaining after isoelectric precipitation of 10 or 20 per cent suspensions of fixed virus brain was also tested for its immunizing properties. For this purpose, the precipitate was re-suspended in the volume of normal saline solution required to produce a total protein content equal to, or half, that of 5 per cent phenol vaccine and the resultant suspension was adjusted to pH 7.

Both *street* and *fixed* virus were used for the test inoculum in animal experiments. The strain of *street* virus used was isolated from the brain of a known rabid jackal.

It was passaged in rabbits and was of high virulence. In all cases where *street* virus was used, the test dose was administered into the deep neck muscles immediately before antirabic treatment was commenced. The test dose of *fixed* virus (Paris strain) was administered subdurally 21 days after the completion of the adopted course of antirabic treatment in all cases. All animals used in the experiments were kept under observation for 6 months after the administration of the test dose of virus.

RESULTS OF EXPERIMENTS

Experiment I—Two groups, each of 10 guinea-pigs, were treated with phenol vaccine and clear vaccine respectively. A third group of 10 animals was untreated. The results are summarized in Table II—

TABLE II

Test dose of <i>street</i> virus	TREATMENT		Number of guinea pigs ^a used	Number which died from rabies *	Number which survived for 6 months	Average incubation period in days
	Dosage and route of administration	Total protein in grammes per 100 c c				
0.5 c c of 10 per cent suspension of <i>street</i> virus brain injected into deep neck muscles immediately prior to commencement of treatment	0.5 c c of 5 per cent phenol vaccine subcutaneously for 7 consecutive days	0.4	10	8	2	16
	0.5 c c of clear vaccine (10 per cent suspension) subcutaneously for 7 consecutive days	0.2	10	10		26
	Untreated		10	0	1	24

* Proved by microscopical examination of brain.

The very high mortality from rabies in all groups was taken to indicate that the test dose was too severe and the experiment was repeated with a reduced test dose.

Experiment II—In addition to the repetition of experiment I using a reduced test dose of *street* virus, this experiment included a group of 10 guinea-pigs treated with re-suspended precipitate. The results are summarized in Table III —

TABLE III

Test dose of <i>street</i> virus	TREATMENT		Number of guinea pigs treated	Number which died from rabies *	Number which survived for 6 months	Average incubation period in days
	Dosage and route of administration	Total protein in grammes per 100 c c				
0.5 c c of 1 per cent suspension of <i>street</i> virus brain injected into deep neck muscles immediately prior to the commencement of treatment	0.5 c c phenol vaccine subcutaneously for 7 consecutive days	0.4	10	5	5	16
	0.5 c c clear vaccine (10 per cent suspension) subcutaneously for 7 consecutive days	0.2	10	5	5	21
	0.5 c c re-suspended precipitate subcutaneously for 7 consecutive days	0.4	10	7	3	17
	Untreated		20	19	1	18

* Proved by microscopical examination of brain

The results of this experiment indicate that, with the reduced test dose, only 5 per cent of the untreated animals survived, while half of the animals treated with phenol or clear vaccine survived. The results obtained with re-suspended precipitate were less satisfactory.

Experiment III—In this experiment, two groups, each of 50 guinea-pigs, were treated with phenol vaccine and clear vaccine respectively. A third group of 50 animals was untreated. The test dose was similar to that used in experiment II.

The total protein content of the clear
of the phenol vaccine The results are

substantiating those observed in
is the test dose The immu-
20 per cent suspensions appeared
vaccine Some evidence was ob-
accine might give at least as
ion Re-suspended precipitate

Table

Test dose of <i>street</i> virus	TREATMENT	
	Dosage and route of administration	Total p. in grams per 100 c.c.
0.5 c.c. of 1 per cent suspension of <i>street</i> virus brain injected into deep neck muscles imme- diately prior to commencement of treatment	0.5 c.c. phenol vaccine sub- cutaneously for 7 consecu- tive days	0.4
	0.5 c.c. clear vaccine (20 per cent sus- pension) sub- cutaneously for 7 consecu- tive days	0.4
	Untreated	

* Proved by microscopical examination.

The results of this experiment were similar to
II except that the mortality from rabies in both
was appreciably lower

For experiments II and III combined, the
70 untreated animals was 83 per cent, among 60
whole phenol vaccine 33 per cent, and among 60 ar-

Experiment IV—Guinea-pigs which had received
to those described in previous experiments were injected
dose of Paris fixed virus which was administered in 10
completion of treatment The results of these experi-
Table V—

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described above is apparently
experimental animals in large
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other 17 c.c. intrathecally

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TABLE V

Test dose of fixed virus	TREATMENT		GUINEA PIGS TREATED (DENOMINATORS) AND DEATHS FROM RABIES (NUMERATORS) AFTER TEST DOSE OF fixed VIRUS FROM SUSPENSIONS AS UNDER				TOTAL	
	Dosage and route of administration	Protein content in grammes per 100 c c	Supernatant from 1 per cent suspension	Supernatant from 0.1 per cent suspension	Supernatant from 0.01 per cent suspension	Observed	Deaths from rabies	
0.2 c c of clear supernatant resulting after centrifugation of suspensions (varying dilutions) of fixed virus rabbit brain injected subcutaneously 21 days after completion of treatment	0.5 c c phenol vaccine subcutaneously for 7 consecutive days	0.4	2/3	1/3	0/3	18	7	
	0.5 c c clear vaccine subcutaneously for 7 consecutive days	0.2	2/3	1/3	1/3	18	7	
	0.5 c c clear vaccine intraperitoneally for 7 consecutive days	0.4	2/3	1/3	0/3	9	3	
	0.5 c c re suspended precipitate subcutaneously for 7 consecutive days	0.4	3/3	2/3	1/3	18	13	
	Untreated	0.2	6/6	5/6	2/6	18	13	

The results obtained may be regarded as substantiating those observed in previous experiments in which *street* virus was used as the test dose. The immunizing value of the clear vaccine prepared from 10 or 20 per cent suspensions appeared to be as good as that of 5 per cent whole phenol vaccine. Some evidence was obtained that the intraperitoneal injection of clear vaccine might give at least as good results as those obtained by subcutaneous injection. Re-suspended precipitate appeared to have little or no immunizing value.

DISCUSSION OF RESULTS

The clear vaccine prepared in the manner described above is apparently free from toxicity even when administered to experimental animals in large doses. In the course of the experiments referred to in the tables animals treated with clear vaccine showed none of the redness and induration at the site of injection frequently observed after administration of phenol vaccine. Doses up to 10 c.c. clear vaccine for 14 consecutive days produced no demonstrable toxic manifestations when administered intravenously or intraperitoneally to rabbits, and sheep receiving double this dosage showed no toxic effects. Clear vaccine has also been administered to human patients suffering from rabies. No untoward effects were observed in two such patients one of whom received 12 c.c. intravenously and the other 17 c.c. intrathecally and 10 c.c. intravenously.

Under the conditions of the experiments described above, it would appear that clear vaccine prepared by isoelectric precipitation of 10 or 20 per cent suspensions of *fixed* virus brain has approximately the same immunizing value as 5 per cent phenol vaccine prepared by Semple's method. This was found to be the case when the total protein content of clear vaccine was equal to, or half, that of phenol vaccine. It would appear to be possible to eliminate much of the nerve tissue from brain suspensions and to obtain a water-clear vaccine the immunizing properties of which do not appear to be inferior to ordinary 5 per cent phenol vaccine which contains all of these substances. This is substantiated by the observation that the precipitate resulting after isoelectric precipitation is unsatisfactory as an immunizing agent even when the total protein content of re-suspended precipitate is equal to that of 5 per cent whole phenol vaccine.

Since commencing these studies a report of a similar investigation has been published by Behrens *et al* (1939). Using a somewhat different technique, these authors succeeded in producing 'water-clear suspensions of rabies virus having satisfactory antigenic properties'. They also observed that 'vaccines made from precipitates obtained from purified rabic emulsions are not satisfactory as immunizing agents'. The work described in this paper confirms the observations of Behrens *et al* (*loc cit*) in these respects but uncompleted experiments at present in progress have not so far confirmed their statement that 'intravenous administration of small doses of avirulent purified vaccines seem to be more efficacious than when injected subcutaneously'.

SUMMARY

Clear antirabic vaccine prepared by isoelectric precipitation of 10 or 20 per cent suspensions of *fixed* virus sheep-brain has approximately the same immunizing value as 5 per cent phenol vaccine prepared by Semple's method under the experimental conditions described. The nerve tissue removed by isoelectric precipitation has relatively little immunizing value. The clear vaccine prepared by the method described is apparently free from toxicity.

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STUDIES ON PEPTIC ULCER IN SOUTH INDIA

Part III.

EXPERIMENTAL PRODUCTION OF GASTRO-DUODENAL ULCER

BY

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(From the King Institute, Gundy, Madras)

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In South India, the only experimental work in connection with the causation of peptic ulcer has been that of McCarrison from the Nutritional Laboratories at Coonoor. In his earlier work McCarrison (1912) recorded changes in the duodenum and upper intestinal canal amongst guinea-pigs fed on ascorbutic diet of crushed oats and autoclaved milk. Four animals were used in this experiment. All died within 46 days of the initiation of the experiment. He concluded 'It appears then that congestive, hæmorrhagic, atrophic and necrotic changes in the bowel walls, usually most pronounced in the upper intestine, are common consequences in guinea-pigs on dietaries deficient in accessory food factors'. This experiment was repeated by him. In his second experiment McCarrison (1931a) found 'duodenal ulceration at all stages of the process up to perforation (in one case)'

At the Seventh Congress of the Far Eastern Association of Tropical Medicine held at Calcutta, in 1927, the surgeons in India presented clinical observations based on proved cases of peptic ulcer. Bradfield (1927) and Somervell (1927), from South India, agreed that the diet of the South Indian played an important role in the ætiology of the disease. It was not certain, however, whether it was the low protein and the high carbohydrate nature of the diet, the excessive quantity of irritating chulies and spices or whether it was deficiency of essential accessory food factors which was the causal agent.

McCarrison (1931b) submitted the dietetic factor to the test of experiment. Starting in 1927, he carried out feeding experiments on albino rats with a diet similar to that in common use by the South Indian. One group of 17 animals was fed on

Madrassi or 'rice diet' consisting of rice, chillies, fish and rice-water, and another group of 17 on Travancore or 'tapioca diet' consisting of tapioca, rice, chillies, tamarind, fish and rice-water. The laboratory diet consisting of whole-wheat flour *chapatties* smeared with fresh butter, bread crusts, sprouted Bengal gram, fresh raw cabbages and carrot *ad libitum*, whole milk, etc. was given to the 'stock' which constituted the control group. The freedom of the stock from gastric or duodenal ulcer was established at post-mortem examination of over 600 animals of all ages.

In the 'rice diet' group 6 of the 17 animals showed lesions of the stomach, whereas 8 out of 17 were affected in the second group fed on 'tapioca diet'. McCarrison (1931a) concluded 'Both the "tapioca diet" eaten by the people of South Travancore, and the "rice diet" which is similar in composition to that eaten by many people in the Madras Presidency, are capable of causing gastric ulcer in albino rats. The tapioca diet is the worst of the two'.

Scrutiny of his protocols, however, showed that in this experiment duodenal ulcer never occurred and that duodenitis was seen in only 3 animals out of 34, i.e. in 8.82 per cent. In these 3 animals death occurred from acute infection, i.e. enteritis in R 1509 and pneumonia with pleurisy in R 1507 and R 1531. The lesions in the stomach consisted of hæmorrhages, gastritis and pin-point ulcers.

In view of the association of the lesions in the stomach and acute generalized infection, in man and experimental animals, the above experimental evidence seemed hardly sufficient to justify the conclusions drawn by McCarrison. Secondly, as has been pointed out (Dogra, 1940a) here in South India, the problem is one of a chronic and progressive lesion in the first part of the duodenum and not one of an ulcer of the stomach. Unless lesions reproduced in the animals are similar to those encountered in man deductions from such experiments are not justifiable. Finally, the guinea-pig, albino rat or the rabbit is not a suitable animal for experiments on the gastro-intestinal tract because of the marked difference in the complex biochemical, physiological and neurological influences operating normally on this system in man as compared with those operating on the above-mentioned laboratory animals.

It was, therefore, deemed necessary to conduct further studies on the experimental production of gastro-duodenal ulcer in a more suitable animal to determine the exact rôle, if any, of the South Indian diet in the ætiology of this disease. The work was started in 1938.

MATERIAL AND METHOD

The choice of a laboratory animal suitable for experiments on the digestive tract fell on the common monkey *Macaca radiata*. As a result of a series of radiological investigations of the alimentary tract, after an opaque meal, a close resemblance of the physiological reactions of this system in man and the monkey was established (Dogra, 1940b). Consequently, *M. radiata* has been exclusively used in these experiments.

The animals weighed between 3,632 grammes and 2,270 grammes with an average weight of 2,951 grammes at the commencement of the experiment. They were kept in individual cages under laboratory conditions in Madras. They were separated into three groups according to the diet administered.

The diet was in all respects identical with that in use by the labouring classes in South India. The methods of cooking employed were the same. Two meals a day were given at regular times, the morning meal between the hours of 9 a.m. and 10 a.m. and the evening meal between 4 p.m. and 5 p.m. Weight was recorded once a week and all the animals were so labelled as not to permit any mistakes in the identity of the diet groups. Group *GA* was fed on 'rice diet', consisting of boiled milled rice of average quality, vegetable (chiefly brinjal) curry and pepper water. No green foods or plantains were allowed. The rice and curry were mixed up along with the pepper water, and the animals allowed to take a sufficient quantity. Group *GB* was fed on 'rice diet', the same as above but, in addition, each animal was given two plantains per day and green foods according to season. The third group *GD* was fed on 'tapioca-rice diet' consisting of boiled milled rice of average quality with tapioca curry. The skin of the root was removed and the pulp cut into small pieces to make the curry with. No green foods or plantains were allowed. The control group *GC* was fed on diet consisting of bread, nuts, bran, plantains, etc.

At the commencement of the experiment, early in September 1938, there were 5 animals in each group on the special diets mentioned above. Within 2 to 3 months some animals died in the groups *GD* and *GA*. As soon as fresh animals were available they were added to these groups, thus making a total of 12 animals in each of the two groups *GA* and *GD*. Three animals constituted the control group *GC* and 5 animals in group *GB*.

RESULTS

The animals in the control group *GC* showed complete absence of any gastro-intestinal disorder on laboratory diet. Their weight was maintained. The animals remained alive and well till the close of the experiment. At autopsy they showed no pathological lesion.

In the group *GB*, i.e. animals fed on curry and rice and green foods, only one animal *GB 5* died after 347 days of the commencement of the experiment. At autopsy, it showed slight signs of emaciation but no pathological changes in the stomach or the duodenum. The other 4 animals *GB 1, 2, 3* and *4* maintained weight and showed no signs of gastro-intestinal disorder during life or at autopsy performed 603 days after the commencement of the experiment.

The animals in groups *GA* and *GD* with a total of 12 monkeys in each group fed on rice and tapioca diets respectively continued for 260 days by which time most of the animals had died. All the animals showed marked degree of progressive emaciation. A few showed petechial hæmorrhage and small cell infiltration of the mucous membrane of the stomach and duodenum but on no occasion was an

ulcer produced Orr and Rao (1939) similarly were unable to produce ulcers in rats and dogs by deficient feedings

CONCLUSIONS

From the above experiment it would appear that high spiced 'rice-and-curry diet' supplemented with green foods and plantains administered to monkeys (*M. radiata*) over a period of almost 2 years caused no ulcerative lesion in either the stomach or the duodenum of the animals. The animals maintained weight and showed no signs of deficiency. 'Rice-and-curry' and 'tapioca' diets on the other hand when administered alone, i.e. without green foods, resulted in marked emaciation and inanition of the animals causing death but did not cause any ulcerative lesions in the stomach or the duodenum.

ACKNOWLEDGMENTS

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ESTIMATION OF THE PROXIMATE PRINCIPLES OF FOOD IN A FEW EDIBLES BY CHEMICAL METHODS

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INTRODUCTION

THE first comprehensive list of food values was published by Ranganathan *et al* (1937). Subsequently this table with the addition of a few more items has been appended in Health Bulletin No 23 (1938). The senior author (Mitra, 1938) has also published the food value of 40 common foods of Bihar. The Public Health Department, Punjab (1939), has published the results of analysis of more than 100 different foods consumed locally. Lastly, Saha and Guha (1940) have studied the food value of 13 different kinds of fish grown in Bengal. Yet the list seems to be incomplete for purposes of reference. Those engaged in dietary survey work are still experiencing considerable difficulty in calculating the value of all the different kinds of foodstuffs consumed, in terms of proteins, fats, carbohydrates, minerals, etc. The table of food values pertaining to 380 Chinese edibles, compiled by Read *et al* (1937), has proved useful at times.

METHODS OF ANALYSIS

In the present study the edible portions of each foodstuff have been analysed according to the methods suggested by the Official Association of the Agricultural Chemists (1930). Iron, though an essential mineral element, has not been estimated for reasons critically examined by Ranganathan (1938). Except in rare cases (noted in the Table) the foodstuffs have all been obtained from different parts of the province of Bihar. In every case the local (Hindi in most cases) names have been given along with the English synonyms as far as practicable. The Latin names have also been appended in the Table.

TABLE

Food values of edible portions in grammes per cent

Serial number	Local name of the food stuff (Hindi)	English name	Latin name	Moisture, g	Protein, g	Fat or other extractives, g	Mineral matter, g	Carbohydrates, g	Crude fibre, g	Calcium, g	Phosphorus, g
GRAIN FOODS											
1	Anandi chawal	A type of rice	<i>Oryza sativa</i>	11.64	5.99	0.24	0.44	81.69		0.008	0.078
2	Atta (Wardha)	Whole wheat	<i>Triticum vulgare</i>	12.32	12.95	0.73	1.61	60.41	1.98	0.045	0.309
3	Bajra	Millet	<i>Hordeum vulgare</i>	9.26	9.06	4.40	1.18	74.66	1.44	0.010	0.218
4	Ghanghra	Cow-peas	<i>Vigna catjang</i>	11.65	21.36	1.26	2.85	60.32	2.56	0.078	0.371
5	Janera	Great millet	<i>Sorghum vulgare</i>	11.04	9.16	3.11	1.58	73.33	1.78	0.046	0.323
6	Kabachi sattoo	Cowage seed flour	<i>Mucuna capitata</i>	5.29	28.19	7.04	1.70	55.60	2.18	0.188	0.211
7	Kodo	Millet	<i>Paspalum scrobiculatum</i>	10.26	5.64	1.66	1.03	81.41		0.024	0.103
8	Kutecha rahar	Tender red gram	<i>Cajanus indicus</i>	75.60	6.21	0.68	1.14	13.69	2.68	0.028	0.119
9	Kusum ka bia	Kusumbh seeds	<i>Carthamus tinctorius</i>	5.47	13.47	25.57	2.61	17.97	34.91	0.236	0.823
10	Kurthi sattoo	Horse gram flour	<i>Dolichos biflorus</i>	6.74	23.28	1.30	3.68	59.74	5.26	0.340	0.258
11	Mota chawal	Coarse rice	<i>Oryza sativa</i>	9.67	7.57	0.62	1.55	80.59		0.010	0.140
12	Purana chawal (Wardha)	Rice with complete aleurone layer	" "	11.98	7.10	2.50	1.51	76.91		0.012	0.264
13	Sutari	Inferior gram	<i>Phaseolus calcaratus</i>	9.63	21.47	0.30	3.45	60.97	4.18	0.302	0.297

[illegible]

TABLE—*contd*

Food values of edible portions in grammes per cent

Serial number	Local name of the food-stuff (Hindi)	English name	Latin name	Moisture, g	Protein, g	Fat or other extraneous, g	Mineral matter, g	Carbohydrates, g	Crude fibre, g	Calcium, g	Phosphorus, g
<i>Fruits—contd</i>											
34	Am, Sipra	Mango	<i>Mangifera indica</i>	73.88	0.63	0.10	0.42	24.27	0.70	0.011	0.002
35	" Bijoo	"	"	81.87	0.86	0.10	0.45	15.76	0.96	0.012	0.003
36	" Bombai	"	"	76.52	0.75	0.19	0.46	21.22	0.86	0.014	0.019
37	" Mithua (Patna)	"	"	81.77	0.99	0.15	0.49	15.87	0.73	0.011	0.016
38	" Jardalu (Bhagalpur)	"	"	80.75	0.58	0.13	0.40	17.35	0.79	0.010	0.013
39	" Gulabkhas (Bhagalpur)	"	"	82.02	0.56	0.17	0.54	15.98	0.73	0.016	0.014
40	Anra	Hog plum	<i>Spondias mangifera</i>	89.47	0.71	0.72	0.54	0.95	1.61	0.112	0.019
41	Bayr	Indian plum	<i>Zizyphus jujuba</i>	85.13	0.64	0.12	0.43	13.02	0.66	0.019	0.031
42	Barhai (ripe)	Monkey fruit	<i>Artocarpus lakoocha</i>	67.58	1.61	0.99	1.52	24.87	3.43	0.051	0.025
43	Bel	Wood apple	<i>Ægle marmelos</i>	64.57	2.31	0.05	1.32	30.14	1.60	0.043	0.042
44	Fleshy peduncle of Huji badam	Cashew apple	<i>Anacardium occidentale</i>	86.18	0.81	0.60	0.37	11.14	0.90	0.004	0.021
45	Jalsa		<i>Grewia asiatica</i>	74.39	1.43	0.25	1.88	20.13	1.92	0.117	0.059
46	Gab		<i>Diospyros embryopteris</i>	69.59	0.42	0.09	0.78	27.58	1.54	0.058	0.027

47	Jamun (big)	Blackberry	<i>Eugenia jambolana</i>	87.93	0.38	0.24	0.28	10.90	0.27	0.008	0.003
48	Kamrak	Bilimbi	<i>Averrhoa carambola</i>	91.86	0.08	0.11	0.35	6.20	0.80	0.004	0.011
49	Kend		<i>Diospyros melanoxylon</i>	78.77	0.74	0.19	0.03	18.95	0.72	0.020	0.009
50	Khirni		<i>Memecopa licantra</i>	72.52	0.79	1.11	0.85	21.95	2.78	0.013	0.042
51	Kosaur		<i>Pachyphus angulata</i>	80.18	1.61	0.10	0.51	16.08	0.62	0.011	0.015
52	Kharbuja (round variety)	Musk melon	<i>Cucurbita melo</i>	90.33	1.11	0.14	0.43	1.09		0.009	0.026
53	Lichis	Lichi	<i>Nephelium litchi</i>	84.07	0.09	0.13	0.51	14.11	0.19	0.004	0.031
54	Perar		<i>Randia uliginosa</i>	81.07	0.97	0.15	0.65	12.67	3.89	0.033	0.013
55	Paniala		<i>Flacoutia cataphracta</i>	77.67	0.52	0.09	0.80	19.89	1.03	0.043	0.025
56	Tar ka pheda	Ripe palmyra fruit (mesocarp)	<i>Borassus flabellifer</i>	77.22	0.67	0.15	0.08	20.68		0.009	0.033
57	Tar ka koa		" "	92.60	0.64	0.10	0.20	6.20	0.11	0.005	0.016
58	Tarbuja	Water melon	<i>Citrullus vulgaris</i>	91.33	0.68	0.14	0.24	4.61		0.003	0.003
MISCELLANEOUS											
59	Coco nut gur (Cochin State)	Coco nut palm jaggery	<i>Cocos nucifera</i>	10.32	0.09	0.15	5.04	83.53		1.638	0.062
60	Khajni gur (Bengal)	Date palm jaggery	<i>Phoenix dactylifera</i>	9.01	1.46	0.26	2.60	80.07		0.363	0.062
61	Khosa (pure milk)	Thokoned cow's milk		27.87	16.47	27.08	3.65	27.33		0.821	0.458
62	Khosa (skimmed milk)	" "		47.44	19.28	1.13	4.08	28.02		0.858	0.565
63	Mahua	Dried flowers of mahua	<i>Bassia latifolia</i>	19.30	5.00	0.19	2.53	70.95	2.03	0.117	0.127
64	Postdana	Poppy seeds	<i>Papaver somniferum</i>	4.26	21.72	10.28	9.87	36.83	8.04	1.584	0.432
65	Sago gur (Bombay)	Sago palm jaggery	<i>Metrexylon sago</i>	9.16	2.28	0.11	3.66	84.79	"	1.252	0.372

TABLE—*concd*

Food values of edible portions in grammes per cent

Serial number	Local name of the food stuff (Hindi)	English name	Latin name	Moisture, g	Protein, g	Fat or other extrac- tives, g	Mineral matter, g	Carbohydrates, g	Crude fibre, g	Calcium, g	Phosphorus, g
<i>MISCELLANEOUS—concd</i>											
66	Tar gur	Fan palm jaggery	<i>Borassus flabellifer</i>	8.61	1.03	0.08	1.81	88.47		0.225	0.044
67	Tissoure	Made of black gram and linseed		15.49	14.44	22.02	7.28	26.27	14.50	0.250	0.438
<i>VEGETABLES</i>											
68	Bakala, green		<i>Vicia faba</i>	77.56	5.63	0.30	1.29	11.57	3.65	0.111	0.149
69	Boro	Cow-pea pods (white)	<i>Vigna catuag</i>	85.86	2.55	0.16	0.80	8.65	1.98	0.063	0.043
70	Chathail or kheksa (big)	Hilly variety	<i>Momordica cochinchinen- sis</i>	90.43	0.58	0.08	0.90	6.44	1.57	0.027	0.038
71	Chaltha		<i>Dillenia indica</i>	82.31	0.77	0.21	0.84	13.40	2.47	0.016	0.026
72	Green mango (Maldia variety)	Mangoes	<i>Mangifera indica</i>	82.97	0.57	0.09	0.29	14.18	1.90	0.010	0.013

73	Gullar	Figs	<i>Ficus hispida</i>	83.32	1.50	0.48	1.40	9.75	3.56	0.155	0.038
74	Khamneelu		<i>Dioscorea alata</i> var <i>glo- bosa</i>	79.68	1.27	0.07	0.82	17.82	0.44	0.021	0.041
75	Kuteha papita	Green papaya	<i>Carica papaya</i>	91.38	0.02	0.10	0.53	6.20	0.87	0.034	0.064
76	Kuteha barhar	Monkey fruit (green)	<i>Artocarpus lakoocha</i>	89.30	1.50	1.15	1.11	11.91	2.88	0.067	0.025
77	Latarn bin	Vegetable	<i>Dioscorea alata</i>	67.64	2.54	0.17	1.37	25.96	2.32	0.014	0.070
78	Latarn	Tuber	" "	72.87	2.52	0.11	1.06	22.75	0.69	0.008	0.070
79	Raharin som	Cluster beans	<i>Cymopsis psoraleoides</i>	82.44	3.71	0.17	1.48	9.81	2.39	0.204	0.050
80	Roma	Cow pea pods (red)	<i>Vigna catuag</i>	81.30	4.27	0.30	0.86	11.30	1.97	0.090	0.058

DISCUSSION

Grain foods—Among the 13 kinds of grain food analysed sutari grains and kusumb seeds were found to be comparatively rich in calcium. Sutari is a kind of inferior grain grown in the district of Santal Parganas. Kusumb trees (small shrubs) are grown all over the province. The seeds are baked and fried dry and eaten with puffed rice after peeling off the skin. Purana chawal from Wardha consisted of shelled rice grains with the pericarp intact. Ghanghra grows abundantly in Chota Nagpur division and the Santal Parganas district only. It is used as a staple food. Green pods of rahar are consumed by the aboriginal children. The flour from kabach seeds are consumed by aboriginals in Chota Nagpur when family store of grains get exhausted.

Vegetables—The Indian figs, cluster beans and bakla are fairly good sources of calcium.

Flesh foods—The samples of dried fish were purchased from Santal Parganas. The use of this foodstuff is confined to a limited area in the province. Higher class Hindus usually do not consume it but in villages where aboriginals predominate it is freely partaken by the former class. All the five varieties examined were found to be rich in all the essential dietary principles.

Ghongha or snails are consumed by aboriginal or semi-aboriginal tribes.

Miscellaneous—Tissourie consists of mashed and dried pulp of black gram with a thick coating of linseed. All these small cakes are fried in oil before serving out in plates. Postdana or poppy seeds seem to be an extremely rich source of mineral matter, particularly of calcium. It is used in curry, condiments and danawri (similar to tissourie). The high mineral content of the four samples of jaggery is due to addition of slaked lime in varying concentration in the samples of fresh juice to arrest fermentation. The heavily limed juice is then thickened to make jaggery.

SUMMARY

Thirteen kinds of grain foods, 18 kinds of flesh foods, 27 kinds of fruits, 9 kinds of miscellaneous foods and 13 kinds of vegetables consumed in Bihar have been analysed.

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those obtained by a biological method depending on the cure of black-tongue in dogs, but have pointed out two possible sources of error in the former, due to (a) incomplete extraction of nicotinic acid and its derivatives and (b) the possible loss of nicotinic acid during the decolorization of extracts

In view of these criticisms a further study of the technique of the test has been undertaken

EXPERIMENTAL

The work to be described falls into two parts —

A The colorimetric procedure and some conditions modifying it

B Its application to food extracts prepared by different methods of extraction and hydrolysis

A The colorimetric procedure—According to the author's method, the reaction with cyanogen bromide and aqueous aniline takes place in the cold. In certain modifications preliminary heating of the test solution with CNBr is resorted to. Kodicek (*loc cit*) has applied the test to alcoholic solutions, and has reported that p-aminoacetophenone gives a deeper colour than aniline or metol. The effect on the colour reaction of these different methods of procedure and of the use of different aromatic amines—aniline, p-aminoacetophenone and β -naphthylamine, and metol—has been studied

Reagents required —

(1) *Standard nicotinic acid (strong)*—One ml = 1 mg, dissolved in N/100 HCl and kept in a frigidaire

(2) *Standard nicotinic acid (dilute)*—One ml = 10 μ g. Prepared as required by diluting 0.5 ml of solution (1) to 50 ml with distilled water, after neutralizing with 0.5 ml of N/100 NaOH

(3) *Standard nicotinic acid (dilute)*—One ml = 10 μ g. Prepared as (2), using alcohol instead of distilled water

(4) *Cyanogen bromide solution*—Prepared as required by decolorizing freshly prepared ice-cold saturated bromine water by the gradual addition of 10 per cent potassium or sodium cyanide

(5) *Aromatic amine solutions* —

Aniline solutions (re-distilled aniline should be used)—

(a) *Aqueous aniline (2 per cent)*—One ml of aniline in 50 ml of water

(b) *Aqueous aniline hydrochloride (10 per cent)*—Aniline 2.5 ml and conc HCl 2.5 ml plus water to make up 25 ml

(c) *Aqueous aniline hydrochloride (5 per cent)*—Aniline 1 ml and conc HCl 6 ml plus water to make up 20 ml

(d) *Alcoholic aniline hydrochloride (10 per cent)*—Aniline 2.5 ml and conc HCl 2.5 ml plus alcohol to 25 ml

p-aminoacetophenone solutions—

(e) *Alcoholic p*-aminoacetophenone (0.5 per cent)—0.1 g dissolved in 20 ml of alcohol

(f) *Aqueous p*-aminoacetophenone hydrochloride (5 per cent)—One g *p*-aminoacetophenone and conc HCl 6 ml plus distilled water to make up 20 ml

 β -naphthylamine solutions—

(g) *Alcoholic β -naphthylamine* (0.25 per cent)— β -naphthylamine 0.1 g dissolved in 40 ml of alcohol

(h) *Aqueous β -naphthylamine hydrochloride* (1.4 per cent)— β -naphthylamine 0.7 g and conc HCl 10 ml made up to 50 ml with water

(i) *Alcoholic β -naphthylamine hydrochloride* (1.4 per cent)— β -naphthylamine 0.7 g and conc HCl 10 ml made up to 50 ml with alcohol

(j) *Alcoholic β -naphthylamine hydrochloride* (0.8 per cent)— β -naphthylamine 0.4 g and conc HCl 2.4 ml made up to 50 ml with alcohol

(k) *Aqueous metol* (5 per cent)—Metol 2.5 g dissolved in 50 ml of water

(6) *Ninety-six per cent ethyl alcohol*—Purified by distilling successively over conc H_2SO_4 and sodium hydroxide (60 per cent) to be freed of pyridine bases and aldehydes. Alcohol purified in this manner was used throughout these investigations

(7) *Sodium hydroxide* 10 N

(8) *Sulphuric acid* 10 N

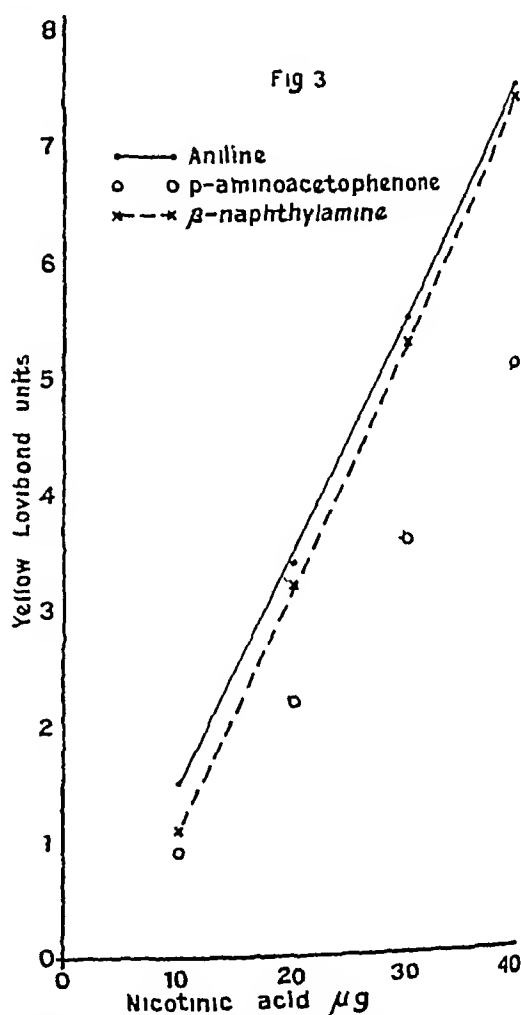
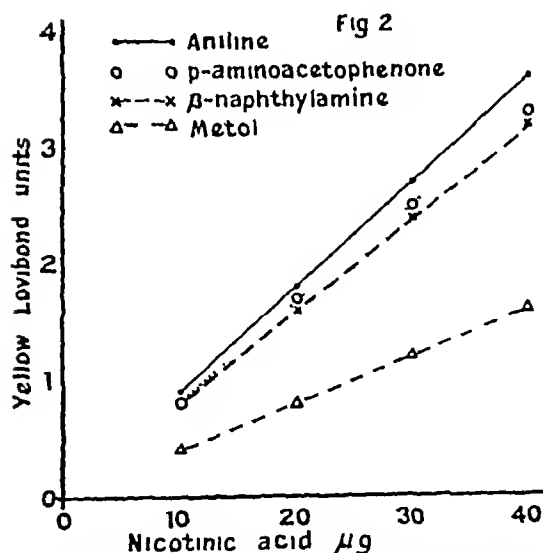
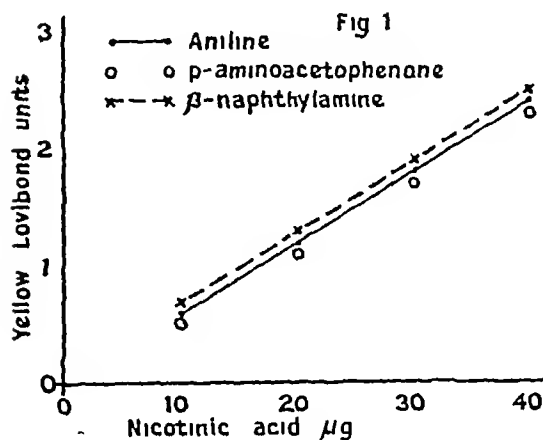
(9) *Sodium acetate* 5 per cent aqueous solution, adjusted to pH 7

Procedure 1 Development of colour in the cold in aqueous solution—Varying amounts of standard nicotinic acid, 10 μg to 40 μg were taken in four 25 ml graduated flasks. Two ml of 5 per cent sodium acetate (pH 7) were then added to all the flasks and each solution diluted to 12 ml with distilled water. Half an ml of 2 per cent aqueous aniline solution (sol 5a) was then added to each, followed by 8 ml of cyanogen bromide. The contents were well mixed and allowed to stand for one minute. Four and a half ml. of aqueous aniline (2 per cent) were then added to each, the mixture being shaken and allowed to stand for one minute as before. The colours were compared in a Lovibond tintometer. Similar experiments were carried out using 0.5 per cent alcoholic *p*-aminoacetophenone and 0.25 per cent β naphthylamine solutions. In performing tests with β naphthylamine, 4.5 ml of alcohol were added at the end of the test, instead of the corresponding amine solution. The results obtained are shown in Fig. 1 of the Graph.

Procedure 2 Development of colour in aqueous solution after preliminary heating with CNBr—Increasing amounts of standard nicotinic acid (10 μg to 40 μg) were taken in four stoppered flasks and each diluted to 10 ml with distilled water. The flasks were placed in a water bath maintained at 70°C to 75°C for 5 minutes. Two ml of cyanogen bromide solution were now added to each. The contents of the flasks were well mixed, by gentle agitation, and allowed to stand at the same temperature for another 4 minutes. The flasks were then transferred to a cold water bath (10°C to 15°C) for 5 minutes. Two ml of 10 per cent aqueous aniline hydrochloride (sol 5b) were added to each, followed by 1 ml of distilled water. The contents of the flasks were well mixed and allowed to stand for 5 minutes in the dark. The colours were compared in the tintometer. Similar experiments were carried out using 0.4 ml. of 5 per cent *p*-aminoacetophenone hydrochloride (sol 5f) and 3 ml of 1.4 per cent β naphthylamine hydrochloride (sol. 5h) instead of aniline, the final volume in each case being maintained at 15 ml by the addition of distilled water if necessary. Colour was also produced using metol, according to the directions of Bandier and Hald (*loc cit*). The results are shown in Fig. 2 of the Graph.

Procedure 3 *Development of colour in alcoholic solution after preliminary heating with cyanogen bromide*—This procedure was similar to procedure 2 described above, except that the test was performed in alcoholic solutions, using the dilute alcoholic solution of standard nicotinic acid. The same amounts of the corresponding alcoholic aniline and β naphthylamine hydrochloride solutions were used in the test. In the case of p aminoacetophenone, the same quantity of the colourless aqueous hydrochloride solution was employed, since the alcoholic solutions were coloured yellow. The results are shown in Fig 3 of the Graph.

GRAPH



Figs 1 to 3 show the relative intensity of the colours produced with pure nicotinic acid using different aromatic amines, according to procedures 1, 2, and 3 respectively.

RESULTS.

Comparison of the colorimetric procedures—Figs 1 to 3 show that aniline gives as intense a colour as p-aminoacetophenone or β -naphthylamine, using the three colorimetric procedures described. The maximum colour with aniline is obtained only when comparatively large amounts are used. In fact, in procedures 2 and 3

metol and p-aminoacetophenone gave less colour than either aniline or β -naphthylamine. But the intensity of the colours can be increased to some extent, by increasing the quantity of the amine. Maximum colour is obtained when the test is performed in alcoholic solutions, using the hydrochloride of the amines. Hence for routine use, procedure 3 is to be preferred to procedures 1 and 2, since the colour obtained is 2 to 3 times as great as in the other two procedures.

Mechanism of the colour reaction—It was found that for the full development of colour in the cold aqueous or alcoholic solutions of the free amines should be used. When a few drops of the amine solutions are added to the test solution containing nicotinic acid, followed by CNBr, colour development takes place immediately (procedure 1). If, on the other hand, aqueous or alcoholic solutions of the amine hydrochloride or sulphate are added before or along with CNBr, no colour is produced in the cold, because the reaction with CNBr does not proceed in the presence of strong mineral acids present in the amine hydrochloride or sulphate. Preliminary heating of nicotinic acid solutions with CNBr, at 70°C to 75°C for 5 minutes, completes the rupture of the pyridine ring. If solutions of amine hydrochlorides or sulphates are then added coupling of the amine with the break-down product takes place immediately, with the development of full colour (procedure 3). Addition of free amine also brings about the same result but the colour obtained is less intense.

The apparent differences in the behaviour of different amines as regards the development of colour observed by certain workers, are not due to differences in their chemical structure. Any aromatic amine or its hydrochloride may be used provided it is colourless and sufficiently soluble in the solvent, under the conditions of the test, the function of the reactive— NH_2 —, OR — NH —groups of the aromatic amines is to combine with the reaction product obtained by the action of CNBr on the pyridine ring.

The results obtained may be summarized as follows: (1) Development of full colour in the cold takes place, when CNBr reacts with nicotinic acid (at pH 7 to 8) in the presence of small quantities of the free aromatic amine. (2) The break-down of the pyridine ring with CNBr can be accomplished in 5 minutes by heating at 70°C to 75°C and colour produced immediately by the addition of the hydrochloride or sulphate of the amine. (3) The addition of strong mineral acids in appreciable amounts to the test solutions, even in the form of aromatic amine hydrochloride or sulphate, before or along with CNBr, completely inhibits the reaction between CNBr and nicotinic acid. (4) The function of any primary or secondary aromatic amine is only to combine with the reaction product formed by the action of CNBr on nicotinic acid.

B Application of colorimetric procedure to food extracts prepared by different methods of extraction and hydrolysis—In the original method of Swaminathan (1938a, b) nicotinic acid and its derivatives occurring in the test materials were extracted with hot water. The amide was hydrolysed and estimated as nicotinic acid. Von Euler *et al* (*loc cit*) used a similar procedure for animal tissues. Bandier

330 *Cyanogen Bromide Method of Estimating Nicotinic Acid*

and Hald (*loc cit*) and Bandier (1939) subjected the whole test material to alkaline hydrolysis and reported values similar to those of Swaminathan (*loc cit*) for animal tissues and yeast. More recently, Kodicek (*loc cit*) has used different methods of extraction and hydrolysis in the preparation of extracts of test materials and estimated the nicotinic acid present in such extracts using the cyanogen bromide p-aminoacetophenone reagent. He reported that the values obtained varied with the method of extraction and hydrolysis and that the residuum left after water extraction contained some 'chromogen', which apparently behaved like nicotinic acid in giving colour with the reagents. The following alternate methods of extraction and hydrolysis have been used in the preparation of extracts for colorimetric estimation —

(a) *Whole material*

1 Extraction during hydrolysis with aqueous NaOH 2 Extraction during hydrolysis with alcoholic NaOH 3 Extraction during hydrolysis with aqueous H_2SO_4 4 Extraction during hydrolysis with alcoholic H_2SO_4

(b) *Aqueous extract*

5 Old procedure (acid hydrolysis and decolorization with charcoal) 6 Hydrolysis with alcoholic NaOH 7 Hydrolysis with alcoholic H_2SO_4

(c) *Residuum left after water extraction*

8 Hydrolysis with aqueous NaOH 9 Hydrolysis with alcoholic NaOH
10 Hydrolysis with aqueous H_2SO_4 11 Hydrolysis with alcoholic H_2SO_4

Details of the different processes used are given below —

(a) *Whole material*

Method 1 Extraction during hydrolysis with aqueous NaOH (8 per cent) — A suitable quantity of the finely powdered test material, containing 100 μg to 500 μg nicotinic acid (the quantities used are indicated in Table I) was suspended in 20 ml of distilled water in a 200 ml. Erlenmeyer flask. Five ml of 10 N NaOH were added and the mixture well stirred with a glass rod (which is not removed). It was then heated for one hour in a steam bath, a glass funnel being used to prevent excessive evaporation. The biologically active derivatives of nicotinic acid, viz the amide and coenzymes I and II, are converted by this procedure into nicotinic acid. The mixture was cooled. One hundred and fifty ml of alcohol were then added and the contents of the flask were well stirred. The precipitate was removed by centrifuging and washed once with 50 ml of alcohol. The combined alcoholic extracts were then neutralized to pH 7 by the addition of 10 N H_2SO_4 , and filtered at the pump. The residue in the filter paper was washed once with 20 ml of alcohol. The extract was finally made up to 250 ml with alcohol. Ten ml aliquots were used for colorimetric estimation of nicotinic acid, using procedure 3.

Method 2 Extraction during hydrolysis with alcoholic NaOH (8 per cent) — A suitable quantity of the test material (1 g to 10 g) was suspended in 40 ml of alcohol, in an Erlenmeyer flask. Ten ml of 10 N NaOH were then added, the contents being well mixed by gentle rotation. The mixture was then heated under reflux for one hour on a steam-bath and allowed to cool. The rest of the procedure was the same as described under method 1.

Method 3 *Extraction during hydrolysis with aqueous H_2SO_4 (2 N)*—The procedure employed was similar to method 1, except that 5 ml of 10 N H_2SO_4 were used for hydrolysis, instead of 5 ml of 10 N NaOH

Method 4 *Extraction during hydrolysis with alcoholic H_2SO_4* —The test material was suspended in 40 ml of ethyl alcohol. Ten ml of 10 N H_2SO_4 were then added. The mixture was heated under reflux for one hour on a steam bath, with occasional shaking. The rest of the procedure was similar to method 3

(b) *Hydrolysis of aqueous extracts of test materials*

A known quantity of the test material (1 g to 10 g) was suspended in 100 to 200 ml of distilled water. The mixture was heated in a water bath ($75^\circ C$ to $80^\circ C$) for 20 minutes, being constantly stirred. It was allowed to cool and the extract separated by centrifuging. The residue was extracted twice in the same manner, and the extracts pooled. Three such extracts were prepared from equal quantity of the same test material.

Method 5 *Old procedure*—Solid lead acetate (1 g to 4 g) was added to the extract, prepared as above, and the procedure described before was then followed (Swaminathan, 1938b). The nicotinic acid present was estimated by using the colorimetric procedure 1, with aniline.

Method 6 *Hydrolysis of the aqueous extract with alcoholic NaOH*—The aqueous extract prepared as described above was concentrated to a small bulk (20 ml) on a water bath and transferring to an Erlenmeyer flask, by washing down with 20 ml of alcohol. Ten ml of 10 N NaOH were then added and the mixture was heated under reflux for one hour on a steam bath. The rest of the procedure was as under method 2.

Method 7 *Hydrolysis of the aqueous extract with alcoholic H_2SO_4* —The aqueous extract prepared as described above was concentrated to a small bulk (20 ml) on a water bath and was transferred to an Erlenmeyer flask by washing down with 30 ml of alcohol. Ten ml of 10 N H_2SO_4 were then added. The mixture was then heated under reflux for one hour. The rest of the procedure was as under method 4.

(c) *Residuum left after water extraction*

Method 8 *Hydrolysis with aqueous NaOH*—The residuum left in the centrifuge tubes after aqueous extraction of the test material was transferred to an Erlenmeyer flask, by washing down with 40 ml of water. Ten ml of 10 N NaOH were added and the mixture was heated in a steam bath for one hour. Fifty ml of water were now added. The mixture was cooled, neutralized to pH 7 by the addition of 10 N H_2SO_4 , and centrifuged. The residue was washed once with 50 ml of water. The mixed centrifugate was evaporated to above 20 ml on a water bath. One hundred and fifty ml of alcohol were now added. The precipitate was removed by centrifuging and was washed once with 30 ml of alcohol. The combined alcoholic extract was readjusted to pH 7, filtered and made up to 200 ml. with alcohol. Ten ml. aliquots were used for the colorimetric estimation of any nicotinic acid that might be present, using procedure 3.

Method 9 *Hydrolysis with alcoholic NaOH*—The residuum left after water extraction was washed down with 40 ml of alcohol into an Erlenmeyer flask. Ten ml of 10 N NaOH were then added. The rest of the procedure was similar to that described under method 2.

Method 10 *Hydrolysis with aqueous H_2SO_4* —The procedure followed was exactly similar to that described under method 8, except that 10 ml of 10 N H_2SO_4 was used for hydrolysis instead of NaOH.

Method 11 *Hydrolysis with alcoholic H_2SO_4* —The residuum left after water extraction was washed down into an Erlenmeyer flask with 40 ml of alcohol. Ten ml of 10 N H_2SO_4 were added. The rest of the procedure was similar to that described under method 4.

The nicotinic acid in the extracts was estimated using a Klett colorimeter. A 'blank' estimation was carried out with each solution in the same manner, with the exception that distilled water was added instead of cyanogen bromide. The values obtained for the 'blanks' were subtracted from the 'total' values to get the 'true' values.

The results obtained with ten foodstuffs, using the different methods of extraction and hydrolysis and different aromatic amines, are shown in Table I —

The nicotinic acid content of certain foodstuffs as determined by various methods

Sample number	Foodstuff	Amount used (g)	Amine used	WHOLE MATERIAL			
				Aqueous NaOH	Alcoholic NaOH	Aqueous H ₂ SO ₄	Alcoholic H ₂ SO ₄
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
1	Maize, whole, white	10 0	Aniline 1	1 6	1 7	1 7	1 7
			" 2	1 5	1 6	1 7	1 6
			p aminoacetophenone	1 8	1 3	0 5	1 1
			β naphthylamine 1	1 6	1 7	1 7	1 8
			" 2	1 5	1 5	1 6	1 5
2	Maize, whole, white	10 0	Aniline 1	1 3	1 3	1 2	1 2
			" 2	1 3	1 2	1 2	1 1
			p aminoacetophenone	1 2	1 1	0 4	0 7
			β naphthylamine 1	1 3	1 3	1 2	1 4
			" 2	1 2	1 2	1 1	1 2
3	Maize, whole, yellow (Rumania)	10 0	Aniline 1	1 4	1 5	1 5	1 5
			" 2	1 3	1 5	1 4	1 3
			p aminoacetophenone	1 3	1 4	0 8	1 0
			β naphthylamine 1	1 4	1 4	1 5	1 4
			" 2	1 3	1 4	1 4	1 3
4	Wheat, whole	10 0	Aniline 1	4 0	3 9	3 6	3 5
			" 2	4 0	4 0	3 8	3 4
			p aminoacetophenone	3 9	3 5	1 3	1 2
			β naphthylamine 1	4 0	4 0	3 6	3 4
			" 2	3 9	3 8	3 7	3 5
5	Rice, raw, milled	10 0	Aniline 1	1 6	1 5	1 4	1 4
			" 2	1 5	1 4	1 3	1 3
			p aminoacetophenone	1 5	1 5	0 9	0 8
			β naphthylamine 1	1 6	1 5	1 4	1 4
			" 2	1 4	1 4	1 3	1 4

Column 4—Aniline 1=2 ml of 10 per cent alcoholic
 Aniline 2=0 4 ml of 5 per cent aqueous
 p aminoacetophenone=0 4 ml of 5 per
 β naphthylamine 1=3 ml of 1 4 per cent
 β naphthylamine 2=3 ml of 0 8 per cent

Column 9—Nicotinic acid was estimated using 2 per

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of extraction and hydrolysis using different aromatic amines (mg /100 g)

AQUEOUS EXTRACT			RESIDUE LEFT AFTER AQUEOUS EXTRACTION			
Original procedure	Alcoholic NaOH	Alcoholic H ₂ SO ₄	Aqueous NaOH	Alcoholic NaOH	Aqueous H ₂ SO ₄	Alcoholic H ₂ SO ₄
(9)	(10)	(11)	(12)	(13)	(14)	(15)
17	17	17	0	0	0	0
	16	16	0	0	0	0
	08	06	0	0	(-11)	(-07)
	16	15	0	0	0	0
	15	16	0	0	0	0
13	14	14	0	0	0	0
	13	14	0	0	0	0
	09	07	(-02)	0	(-08)	(-05)
	13	13	0	0	0	0
	12	13	0	0	0	0
15	14	15	0	0	0	0
	13	14	0	0	0	0
	09	06	(-02)	(-02)	(-09)	(-06)
	15	14	0	0	0	0
	14	13	0	0	0	0
38	37	37	04	03	04	03
	37	35	02	02	02	02
	28	22	02	02	(-09)	(-08)
	36	37	04	04	04	04
	37	38	02	02	02	02
15	16	15	0	0	0	0
	15	13	0	0	0	0
	11	09	0	(-02)	(-04)	(-05)
	15	15	0	0	0	0
	14	04	0	0	0	0

aniline hydrochloride (sol 5d)

aniline hydrochloride (sol 5c)

cent aqueous acid solution (sol 5g)

alcoholic acid solution (sol 5i)

alcoholic acid solution (sol 5j)

cent aqueous aniline according to procedure 1

TABLE

Sample number	Foodstuff	Amount used (g)	Amine used	WHOLE MATERIAL			
				Aqueous NaOH	Alcoholic NaOH	Aqueous H ₂ SO ₄	Alcoholic H ₂ SO ₄
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
6	Rice, parboiled, milled	10 0	Aniline 1	3 2	3 2	3 1	3 0
			" 2	3 0	3 1	3 0	2 9
			p aminoacetophenone	2 9	2 8	1 9	1 8
			β naphthylamine 1	3 1	3 1	3 0	3 0
			" 2	2 9	3 0	2 9	2 9
7	Italian millet	10 0	Aniline 1	0 8	0 8	0 8	0 9
			" 2	0 7	0 7	0 7	0 8
			p aminoacetophenone	0 5	0 4	0 2	0 3
			β naphthylamine 1	0 8	0 7	0 7	0 8
			" 2	0 6	0 6	0 6	0 6
8	Rice polishings, raw	2 0	Aniline 1	28 4	30 3	29 7	30 7
			" 2	26 4	27 8	28 7	28 7
			p aminoacetophenone	20 8	28 6	20 0	24 6
			β naphthylamine 1	28 4	28 4	26 8	30 4
			" 2	26 4	26 1	25 7	29 2
9	Rice polishings, (from parboiled paddy)	5 0	Aniline 1	6 4	6 2	6 0	6 1
			" 2	6 2	6 0	5 8	5 7
			p aminoacetophenone	4 8	4 6	3 6	3 8
			β naphthylamine 1	6 0	6 2	6 0	6 0
			" 2	5 8	5 8	5 8	5 7
10	Yeast, brewer's, dried	1 0	Aniline 1	49 2	49 2	49 2	47 6
			" 2	48 2	46 0	45 2	45 6
			p aminoacetophenone	35 2	30 8	32 0	24 8
			β naphthylamine 1	48 4	48 4	48 4	48 2
			" 2	45 2	44 4	45 2	41 2

Column 4—Aniline 1=2 ml of 10 per cent alcoholic
 Aniline 2=0.4 ml of 5 per cent aqueous
 p aminoacetophenone =0.4 ml of 5 per
 β naphthylamine 1=3 ml of 1.4 per cent
 β naphthylamine 2=3 ml of 0.8 per cent
 Column 9—Nicotinic acid was estimated using 2 per

I—concl'd

AQUEOUS EXTRACT			RESIDUE LEFT AFTER AQUEOUS EXTRACTION			
Original procedure	Alcoholic NaOH	Alcoholic H ₂ SO ₄	Aqueous NaOH	Alcoholic NaOH	Aqueous H ₂ SO ₄	Alcoholic H ₂ SO ₄
(9)	(10)	(11)	(12)	(13)	(14)	(15)
3 0	3 2 3 0 2 0 3 1 3 0	3 1 2 9 1 4 3 1 2 9	0 0 (-0 2) 0 0	0 0 (-0 4) 0 0	0 0 (-1 4) 0 0	0 0 (-1 2) 0 0
0 8	0 8 0 7 0 6 0 8 0 7	0 8 0 8 0 6 0 8 0 7	0 0 (-0 2) 0 0	0 0 (-0 2) 0 0	0 0 (-0 4) 0 0	0 0 (-0 4) 0 0
29 5	30 3 26 4 22 7 26 8 24 2	28 3 26 2 21 7 26 2 21 7	0 2 0 2 (-0 2) 0 2 0 2	0 2 0 2 (-0 2) 0 2 0 2	0 2 0 2 (-0 5) 0 2 0 2	0 2 0 2 (-2 3) 0 2 0 2
6 0	6 2 5 8 4 2 6 0 5 6	6 3 5 7 3 2 6 2 5 9	0 0 (-0 2) 0 0	0 0 (-0 4) 0 0	0 0 (-1 2) 0 0	0 0 (-1 8) 0 0
48 3	46 9 41 3 27 4 47 7 43 1	48 7 45 2 41 3 48 2 43 1	0 0 (-7 2) 0 0	0 0 (-6 9) 0 0	0 0 (-7 5) 0 0	0 0 (-7 2) 0 0

aniline hydrochloride (sol 5d)

aniline hydrochloride (sol 5c)

cent aqueous acid solution (sol 5g)

alcoholic acid solution (sol 5i)

alcoholic acid solution (sol 5j)

cent aqueous aniline according to procedure 1

RESULTS.

(a) *Maize*—The association between maize and pellagra has long been recognized. The diet used by Waisman *et al* (*loc cit*) to produce experimental black-tongue in dogs, which is said to be analogous to human pellagra, contained 71 per cent of whole yellow maize meal. It is natural to assume that maize is devoid of nicotinic acid or contains this factor in very small quantities. Aykroyd and Swaminathan (*loc cit*) showed, however, that the nicotinic acid content of whole maize, which ranged from 1.2 mg to 1.6 mg per cent, was not lower than that of certain other cereals. The results obtained with the varying procedures followed in the present investigation confirm these findings. The nicotinic acid content of three maize samples, using aniline and β -naphthylamine as the aromatic amines, ranged from 1.2 mg and 1.6 mg per cent. Different methods of extraction and hydrolysis yielded almost similar results when aniline and β -naphthylamine were used, but with p-aminoacetophenone the values obtained varied widely. Low values of 0.4 mg to 0.9 mg per cent were obtained when acid hydrolysis of the whole material and alkaline and acid hydrolysis of the aqueous extract were resorted to. Alkaline hydrolysis of the whole material gave higher values (1.2 to 1.7). The lower values obtained with p-aminoacetophenone with certain types of hydrolysis were due to the fact that the blank values were correspondingly higher.

The residuum left after water extraction was subjected to different methods of hydrolysis and its nicotinic acid content estimated using three different amines. When aniline or β -naphthylamine were used, not even a trace of nicotinic acid could be found. On the other hand, with p-aminoacetophenone the blank gave a deeper colour than the test, producing the minus values reported in Table I. These results are not in agreement with those of Kodicek (*loc cit*), who reported that the residuum left after water extraction contained some 'chromogen' which behaved like nicotinic acid with the reagent and that the hydrolysis of the whole material with aqueous or alcoholic NaOH resulted in high values ranging up to 4.5 mg per cent in certain samples.

(b) *Whole wheat*—In a previous investigation (Aykroyd and Swaminathan, *loc cit*) a value of about 5 mg per cent was obtained for whole wheat. A slightly lower value (4.0 mg) was obtained in this investigation for a different sample of whole wheat. Different methods of extraction and hydrolysis had no appreciable effect on the nicotinic acid value when aniline and β -naphthylamine were employed. On the other hand, varying results were obtained with p-aminoacetophenone, for the same reason as in the case of maize. The residuum left after water extraction was subjected to various methods of hydrolysis and tested. Traces of nicotinic acid (0.2 mg to 0.4 mg per cent) were found when aniline and β -naphthylamine were used. With p-aminoacetophenone the alkali-hydrolysed extracts gave small positive values, while the acid-hydrolysed extracts gave minus values.

(c) *Rice*—Aykroyd and Swaminathan (*loc cit*) found that parboiled milled rice contains 2 to 3 times the nicotinic acid present in raw rice milled to the same degree. The figures reported in the present paper for one sample of raw and parboiled milled rice respectively (1.6 mg and 3.0 mg) correspond well with those

reported before. The results obtained on subjecting the samples to various methods of extraction and hydrolysis were similar when aniline and β -naphthylamine were used, but as before varying values were obtained with p-aminoacetophenone. The residuum did not give a positive test whatever methods were employed.

(d) *Rice polishings*—The difference between raw and parboiled milled rice in nicotinic acid content is reflected in the values given by bran from raw and parboiled rice respectively. Raw rice bran was found to be 5 times richer than parboiled rice bran. Similar results have been reported in the case of vitamin B₁ (Aykroyd, Krishnan, Passmore and Sundararajan, 1940). The results of testing bran samples by various methods were similar to those obtained with cereal grains.

(e) *Italian millet*—The very low value (0.6 mg to 0.8 mg per cent) obtained by the cyanogen bromide method in the case of Italian millet is interesting. No other whole cereal grain has given such a low value. Italian millet is consumed in considerable quantities in certain parts of South India, but there is no evidence that pellagra is common in these areas. The various testing methods gave similar values.

(f) *Dried brewer's yeast*—Swaminathan (1938b) reported a value of 57 mg to 60 mg per cent for the nicotinic acid content of a sample of dried brewer's yeast. Figures of the same order have been obtained in later investigations. In the present investigation the values obtained with aniline and β -naphthylamine were nearly the same, irrespective of the method of extraction used, but with p-aminoacetophenone the usual variation resulted.

(g) *Milk*—A value of 10 mg per cent was previously reported by the author for skimmed milk powder (Swaminathan, 1938a). Recently, Waisman *et al* (*loc cit*), using a biological method, obtained a figure of 4 mg to 5 mg per cent for skimmed milk powder. Kodicek (*loc cit*) found values of 0.1 mg to 0.5 mg per cent for liquid cow's milk and values lower than one μ g per ml, for samples of human milk. The values given in Table II are in agreement with those of Kodicek and in contradiction to the value previously reported by the author. The higher value reported previously appears to be the result of an error in calculation.

TABLE II

The nicotinic acid content of milk and milk powder

Milk sample	Nicotinic acid mg /100 g (by acid hydrolysis)
Skimmed milk powder	1.0
'Klm' (whole milk powder)	1.1
Cow's milk, fresh	0.1

(h) *Urine*—It was found that colorimetric estimations on urine could not be carried out in alcoholic solutions, owing to the development of strong red colours which mask the yellow colour given by nicotinic acid. These interfering colours are probably produced by the action of aromatic amine hydrochlorides on certain types of organic compounds (aldehyde or ketonic in nature) present in urine, e.g. furfural, acetaldehyde and acrylic aldehyde, etc (Snell and Snell, 1937). Such side reactions do not occur when estimations are made in aqueous solution, using aqueous aniline (Swaminathan, 1939). Throughout the previous investigation it was found that the 'dilution blank' was the same as the 'aniline blank'. Evidently, application to urine of other procedures described in this paper will lead to difficulties. The author's method (Swaminathan, 1939) has been successfully applied to urine.

(i) *Starch and casein*—The results obtained with foodstuffs using p-aminoacetophenone showed that certain substances are produced during the process of hydrolysis which give a high blank. To investigate this point further, similar tests were carried out with starch and casein. It was found that the products obtained by the hydrolysis of starch interfere in the estimation when p-aminoacetophenone is used. Since no such interference occurs with aniline and β -naphthylamine, under the conditions described, it appears likely that the acetophenone group in p-aminoacetophenone takes part in side reactions in the absence of CNBr.

DISCUSSION.

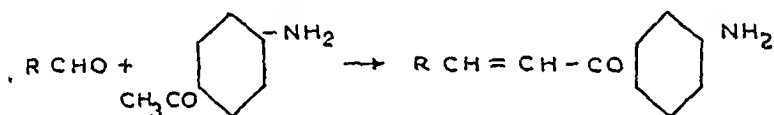
Specificity of the colour reaction—The colour reaction is not specific for nicotinic acid, many other pyridine derivatives which have the α -position free give the reaction. Fortunately, however, two pyridine derivatives widely distributed in biological materials, vitamin B₆ and trigonellin, do not react. Most of the other pyridine compounds giving the colour test can if present be separated from nicotinic acid. Nicotinamide and coenzymes I and II if present can be hydrolysed to nicotinic acid. Bandier (*loc cit*) states that picolinic acid, a biologically inactive isomer of nicotinic acid, does not give the reaction with cyanogen bromide and metol and probably this will apply when aniline is used. The other isomer, isonicotinic acid, which is biologically inactive, has not yet been recognized in biological materials. It thus appears highly unlikely that substances other than nicotinic acid in biological substances interfere with the specificity of the test.

Choice of the aromatic amine—Aniline has several advantages over other amines. It is cheap, almost colourless when freshly distilled and highly soluble in the solvents under the experimental conditions. Further, unlike p-aminoacetophenone, it does not lead to any side reactions in the blank. It gives as good a colour as the other aromatic amines tested. Possibly any primary or secondary aromatic amine could be used in the test, provided it is colourless and sufficiently soluble under the conditions of the test.

The colour obtained with any amine varies with the conditions of the colorimetric procedure. Hence the claim that p-aminoacetophenone gives a deeper

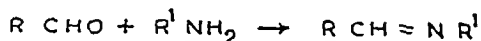
colour than other amines was found to be invalid when a standard colorimetric procedure was followed in the case of each amine

Side reaction of the aromatic amine—According to Melnick and Field (1940b) aniline, when added to the blank, produces an unspecific colour in the absence of CNBr, so that a lower value for nicotinic acid content was obtained. Hence these authors advocate the use of a 'dilution blank' instead of the 'aniline blank'. They have further shown that such difficulties are encountered with urine which has been subjected to acid hydrolysis for half an hour and not with specimens subjected to hydrolysis for 5 hours. The author has not had a similar experience. When colorimetric estimations were made in aqueous solutions the 'aniline blank' was always found to be the same as the 'dilution blank'. When estimations are made on alcoholic solutions the 'aniline blanks' may give higher values than 'dilution blanks', but in such cases there is a corresponding increase in the colour of the test solutions so that the final values obtained are not appreciably affected. Phenomena similar to those reported by Melnick and Field have been observed when p-aminoacetophenone was used and are probably due to the reaction of aldehydic products of sugars with the acetophenone group. The reaction may be represented as follows —



Such reactions take place with great ease in acid alcohol solutions

The reactions occurring in urine when alcoholic solutions are used can be represented by the following equation —



Extraction and hydrolysis of nicotinic acid derivatives from the test materials —

The extraction and hydrolysis of test materials in alcoholic solutions may thus produce substances which occasionally interfere when estimations are made in alcoholic solution, using the amine hydrochloride. Further, hydrolysis of the whole material can be resorted to only in the case of test materials which are almost colourless or have a light yellow colour, but in the case of highly coloured test materials, e.g. certain pulses and vegetables, nicotinic acid and its derivatives should be extracted with water, after which the colouring matter, protein derivatives and starch are removed from the aqueous solution by lead acetate. In general, the results recorded in the present paper support the view that the methods of extraction and hydrolysis previously described by the author give the most satisfactory results.

SUMMARY.

1. Further studies on the cyanogen bromide method of estimating nicotinic acid in biological materials have been made. Special attention has been given to two problems (a) the choice of the aromatic amine and (b) the influence of various

methods of extraction and hydrolysis on the value obtained for nicotinic acid content

2 Three different procedures are described for the development of colour with nicotinic acid using cyanogen bromide and an aromatic amine. Different aromatic amines, viz aniline, p-aminoacetophenone, β -naphthylamine and metol, have been used in the above three processes. It has been shown that aniline has many advantages over other amines, being cheap, highly soluble in the solvents under the experimental conditions, and at the same time giving colours as intense, or more intense, than those given by other aromatic amines.

3 The influence of different methods of extraction and hydrolysis was investigated. Whatever the method, the values obtained with ten foodstuffs were similar when aniline or β -naphthylamine was used. Lower values were usually obtained when p-aminoacetophenone was employed. The acetophenone group was probably responsible for the side reaction. Estimations of nicotinic acid could not be carried out on alcoholic extracts of urine owing to the development of red colours from the interfering substances present.

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NICOTINIC ACID IN BLOOD AND IN URINE

BY

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In a previous communication from this laboratory (Kochhar, 1941) it was recorded that (a) most of the nicotinic acid in blood was present in the red cells and (b) there was little fluctuation in the blood nicotinic acid values in health and disease. Swaminathan (1939) showed that the administration of nicotinic acid to normal subjects resulted in an excretion of about 12 per cent of the amount ingested. Harris and Raymond (1939) recorded still less excretion of nicotinic acid in urine. Thus, most of the ingested nicotinic acid is either retained in the body or excreted in the form of trigonellin, which is not estimated by the method. After the ingestion of nicotinic acid its increased concentration in tissues in the forms of co-enzyme, V-factor, etc. has been observed by various workers (Kohn, 1938, Kohn, Klein and Dann, 1939, Vilter, Vilter and Spies, 1939, Kohn and Klein, 1939).

It is concluded that the rise, if any, in the nicotinic acid content of blood after a test dose will be only temporary, excess of the retained nicotinic acid being eliminated from the blood to bring down the blood level to normal. The present investigation was undertaken to find out the changes in the blood after the administration of a 'test dose' of nicotinic acid and whether continuous intake produces any 'saturation' in the blood. Observations in regard to nicotinic acid elimination by the kidney and its variation after giving 'test doses' were also made on healthy individuals belonging to the wheat-eating population of the Punjab.

METHOD.

1 *Blood*—The details of the method for the estimation of nicotinic acid content of blood have been described in the author's previous communication (Kochhar, 1940)

2 *Urine*—The reagents and the method used for the estimation of nicotinic acid in urine are those described by Swaminathan (1939). The method was modified slightly for routine clinical purpose. In brief it was as follows —

To 10 ml of urine in a 20-ml narrow-bore test-tube were added 2 ml of 40 per cent NaOH. A number of such tubes was taken in a beaker containing water and kept in a boiling water-bath for 3 hours. The mixture after cooling was neutralized by conc HCl, filtered and the residue washed twice with 1 ml of water. The mixed filtrate was made alkaline with 2 drops of 40 per cent NaOH solution and boiled for 15 minutes with 0.5 g of absorbent charcoal. It was filtered hot and the charcoal washed thrice with 2-ml portions of boiling alkaline water. The mixed filtrate was brought to pH 7, made up to a volume and filtered. An aliquot was transferred to a 25-ml flask containing 2 ml alcohol and made up to 12 ml. To it were added 8 ml KCNBr from a burette followed after 3 minutes by aniline solution and the colour developed was matched with a standard prepared with a known amount of nicotinic acid using alcohol, KCNBr and aniline solution. A blank was made in samples which were coloured.

3 *Food*—The method used for the estimation of nicotinic acid in food was a modification of that described by Swaminathan (1938). The conditions for the modified method have been worked out and are given below —

One-fourth of a full meal was transferred to a large mortar and thoroughly ground until a homogeneous paste resulted. In a tared beaker 20 g were taken, covered with lead foil, autoclaved at 110°C for 1 hour to break the cells of uncooked articles and weighed. The content was again triturated to a homogeneous paste in a mortar and 5 g of homogenized paste from the digest were introduced into a 50-ml measuring flask containing water. This was placed in a boiling water-bath for $\frac{1}{2}$ hour, shaken at intervals, cooled, made up to 50 ml, mixed and filtered. In a long test-tube with a mark at 25 ml were taken 20 ml of the filtrate to which 4.8 ml of 0.9 per cent ZnSO_4 and 0.2 ml N/1 NaOH were added, boiled for 5 minutes, cooled, made to the mark and filtered. To 20 ml of this filtrate 2 ml conc HCl were added, the mixture was boiled for 30 minutes, cooled, neutralized, made up to 25-ml volume and filtered. To 20 ml of the filtrate 2 drops of NaOH and 1 g of absorbent charcoal were added, after which the mixture was boiled for 15 minutes, cooled and filtered. The residue was washed thrice with boiling alkaline water. The mixed filtrate was brought to pH 7 (bromothymol blue), made to a known volume, and the aliquots examined colorimetrically as in blood or in urine.

Note—Charcoal treatment was avoided in samples which were colourless.

RESULTS,

1 *Blood*—The results given in Table I show normal value for the nicotinic acid content of blood and its day-to-day variation depending upon the intake of nicotinic acid. In response to a daily oral dose of 200 mg there was a slight gradual increase in the blood nicotinic acid which soon reached its maximum in normal persons and was not altered if the administration of nicotinic acid was continued

TABLE I

Changes in the nicotinic acid content of blood and in urinary output after a continuous intake of 'test doses'

Subjects		NORMAL.		24 HOURS AFTER INTAKE OF 200 MG NICOTINIC ACID				—	After discontinuing nicotinic acid intake	
		March 1940								
		21	26	27	28	29	30			
1	A A	Blood	440	435	440*	460†	490†	500†	µg per cent	
		Urine		7.0	40.6	26.2	23.7	24.2	mg in 24 hours	
2	V S	Blood	284	300	400*	440†	400†		µg per cent	
		Urine		10.8	28.9				mg in 24 hours	
3	D N	Blood	320	320	344	420	468	450†	µg per cent	
		Urine		5.4	28.7	24.7	17.7	14.1	mg in 24 hours	
4	R R	Blood	300	320	390	400	420†		µg per cent	
		Urine		4.5	14.9	9.0	27.8		mg in 24 hours	
5	Kh.	Blood	208	290	320	380§	420	470	µg per cent (continued in Table II)	
		Urine		4.8	11.5	15.5	16.1	20.5	mg in 24 hours	
6	R L	Blood	300	270	320	360	400†	420†	µg per cent	
		Urine		3.0	21.9	11.7	8.4	13.8	mg in 24 hours	
7	B D		310	266	416	640†			µg per cent	
8	Du			240	350†				µg per cent	

* 300 mg of nicotinic acid.

† 100 mg of nicotinic acid

‡ Intake of nicotinic acid was stopped

§ 150 mg of nicotinic acid

Note.—Due to undesirable symptoms the doses of nicotinic acid had to be reduced in some cases

further for a few days. The intake of 3,000 mg of nicotinic acid over a period of 15 days by one normal subject on a wheat diet left this maximum unaffected (Table II). The basal level of the nicotinic acid content of blood appears to be a fair measure of the time during which the maximum is reached. On discontinuing the nicotinic acid intake the nicotinic acid content of blood returned to normal level.

TABLE II

Effects of further administration of nicotinic acid to subject No 5, Table I

Dates in 1940	Nicotinic acid intake, mg	Nicotinic* acid in blood after 24 hours, μ g per cent	Nicotinic acid excretion in urine in 24 hours, mg
March 21 to 30 .	See Table I, subject No 5		
„ 31	200		27.0 (1st 3 hours)
April 1 and 2	200 daily		.
„ 3	200		29.0
„ 4	200	500	
„ 5	200		23.3 (1st 3 hours)
„ 6	200	500	27.4
„ 7 and 8	200 daily		.
„ 9	200	480	20.0
„ 10	<i>Nil</i>		9.7
„ 11	<i>Nil</i>	350	6.8

* Nicotinic acid was given after taking out blood for analysis

In Tables III and IV it is shown that most of the nicotinic acid after its ingestion is excreted in the first 2 hours, and a corresponding rise or 'peak' in the blood content is observed. The subjects under observation were all apparently

healthy individuals but the immediate response to a 'test dose' as measured by the changes in blood and urinary excretion seems to be proportional to the basal blood content or urine output

TABLE III

The immediate effect of a 'test dose' of nicotinic acid on blood nicotinic acid content

Subjects	Nicotinic acid intake, mg	NICOTINIC ACID CONTENT OF BLOOD AFTER					
		0 minute, μg per cent	15 minutes, μg per cent	30 minutes, μg per cent	60 minutes, μg per cent	90 minutes, μg per cent	120 minutes, μg per cent
1	300	420		960	640	560	540
2	300	368	872	1,238	756	408	448
3	300	280	272	620	480	380	360
		0 hour			1 hour	6 hours	24 hours
4	300	435			790	500	440
5	300	300			515	400	400
6	200	320			540	508	344
7	200	320			480	390	390
8	200	290			500	360	320
9	200	270			464	300	320
10	200	266			470		416
11	200	240			400		350

Notes — Meals taken by subject No. 2 was 26 hours before the start of experiment

2 *Urine* — There was a sharp increase in the output of nicotinic acid in urine after oral administration of 200 mg of nicotinic acid (Table I). The increase of output was proportionately high in subjects with a high initial excretion. Unless nicotinic acid deficient subjects are tested in a similar manner, it is difficult to arrive at any conclusion. Swaminathan (1939) has shown that it is possible to detect nicotinic acid deficiency in human beings by the study of the initial excretion and excretion after 'test doses'. The variation in Table I was too small to form the basis of any definite conclusion. It may be that none of the subjects under observation were taking insufficient nicotinic acid in the diet. When the intake (200 mg nicotinic acid) was continued the output became almost constant (Tables I and II).

TABLE IV

The immediate elimination of nicotine acid in urine after 'test dose' of nicotinic acid by mouth

NICOTINIC ACID OUTPUT													
Subjects	Nicotinic acid intake, mg	NORMAL		AFTER ADMINISTRATION OF NICOTINIC ACID IN SAMPLES VOIDED AT						Total output, mg	Per cent excreted during		
		— 60 minutes, mg	0 minute, mg	15 minutes, mg	30 minutes, mg	60 minutes, mg	90 minutes, mg	120 minutes, mg	180 minutes, mg				
		1 hour	1 hour										
1	300		0 35	0 30	2 20	22 03	11 32	6 35	4 70		3 hours	3 hours	
2	300		0 10			25 00	20 40	8 00	8 21		46 90	15 6	
3	300		0 09	0 09	3 60	13 95	12 77	5 08	1 62		61 61++	20 5+	
12	200	0 29	0 20	*	2 70	14 85	*	13 61	2 75		37 11	12 4	
13	200	0 16	0 14	*	2 87	11 29	*	12 00	3 29		33 91	16 9	
14	200	0 21	0 22	*	3 12	7 30	*	7 04	1 29		29 45	14 7	
15											18 75	9 3	
Subject No 5, Table I, 200 mg daily from March 26 to April 6 (12 days)													

Subject No 5, Table I, 200 mg daily from March 26 to April 6 (12 days)

* Urine not voided

Note —1 Subject numbers correspond to the numbers in Table III

2 Meals consumed by subject Nos 2 and 13 were taken 26 and 17 hours respectively before the start of experiment, whereas the last meal taken by other subjects was about 1½ and 2 hours before the experiment

TABLE IV—*concl'd*

NICOTINIC ACID OUTPUT												
Subjects	Nicotinic acid intake, mg	NORMAL		AFTER ADMINISTRATION OF NICOTINIC ACID IN SAMPLES VOIDED AT							Total output, mg	Per cent excreted during
		- 00 minutes, mg	0 minute, mg	15 minutes, mg	30 minutes, mg	60 minutes, mg	90 minutes, mg	120 minutes, mg	180 minutes, mg			
	April 1940			*	*	13.2	*	*	9.2	3.5	26.00	13.0
	3			*	*	15.0	*	*	7.2	1.1	23.30	11.0
	5			*	*	12.5	*	*	8.8	3.1	24.40	12.2
	0				1.31 per cent	0.33 per cent			3.58 per cent	1.4 per cent		14.1 per cent
	AVERAGE											
			11 a m 24 hours				2 p m 3 hours	5 p m 3 hours		11 a m following day 18 hours	24 hours	24 hours
4	300		7.0	*	*	*	34.0	3.5	3.1		43.00	14.5
5	300		10.8	*	*	*	22.0	0.1	0.8		28.0	9.0
6	200		5.4	*	*	*	25.4			3.3	28.7+	14.4+
7	200		4.5	*	*	*	0.7	4.5		3.7	14.0	7.5
8	200		4.8	*	*	*	0.5	2.0		3.0	11.5	5.7
9	200		3.0	*	*	*	11.0	7.5		2.8	21.0	10.0

* Urine not voided

Note —1 Subject numbers correspond to the numbers in Table III

2 Meals consumed by subject Nos 2 and 13 were taken 26 and 17 hours respectively before the start of experiment, whereas the last meal taken by other subjects was about 14 and 2 hours before the experiment

An attempt to show quantitatively the relationship between nicotinic acid intake and its hourly excretion in urine is made in Table IV. The excretion of nicotinic acid in urine during the first 3 hours was about 71 per cent of the total urine excretion in 24 hours. Maximum amount excreted was during the second half of the first hour and the first half of the second hour, being 44.9 per cent and 31 per cent respectively of the total excretion in the first 3 hours. This increased excretion corresponded to the peak observed in the blood.

Table V shows the 3-hourly excretion of nicotinic acid in two individuals on a normal diet as well as fasting. The nicotinic acid content of food was determined experimentally.

TABLE V

Subjects	April 1940	Nicotinic acid intake	NICOTINIC ACID OUTPUT					
			6 a m to 9 a m 3 hours, mg	9 a m to 12 noon 3 hours, mg	12 noon to 3 p m 3 hours, mg	3 p m to 6 p m 3 hours, mg	6 p m to 6 a m 12 hours, mg	TOTAL 24 hours, mg
1	5	Meals at 8 a m and 7 p m (containing 52 mg)	0.71	0.73	0.69	0.61	3.1	5.84
	6	Fast	0.54	0.49	0.51	0.45	1.97	3.96
	7	Two meals (containing 37 mg)						
	8	Meal at 12 noon (containing 19 mg)		0.52	0.68			
2	11	Last meal at 7 p m on April 10		0.58	0.66			

DISCUSSION

The ingestion of nicotinic acid causes an immediate increase in the nicotinic acid content of blood. This increase is temporary and explains for the previous

observation (Kochhar, 1940) that the nicotinic acid content of blood changes very little in health and disease

After a 'test dose' of nicotinic acid the proportional increase in the output of nicotinic acid in urine was high in subjects with a high level of initial excretion. The variation in the normal cases examined was too small to enable even a tentative conclusion to the state of nutrition being formed. This agrees with the conclusion of Swaminathan (1939), Bandier (1939) and Harris and Raymond (*loc cit*) on the excretion of nicotinic acid in urine before and after 'test doses' and of Harris and Ray (1935), Harris and Leong (1936), Harris, Leong and Ungley (1938) in the case of vitamins B and C. The organism's capacity to store nicotinic acid seems to be limited. In urine only a small percentage of the ingested nicotinic acid is excreted in 24 hours, the blood figure also falls in about 2 hours. This suggests that nicotinic acid after its absorption is removed from the blood. Nicotinic acid is a constituent of the red cells (Kochhar, 1941) but whether at the peak time the nicotinic acid excess in blood is present in plasma or in the red cells is yet to be determined.

SUMMARY

1 Oral administration of nicotinic acid produced a sudden temporary rise in the blood content of nicotinic acid. The peak was observed in 30 minutes time.

2 Continuous intake of nicotinic acid resulted in a slight gradual increase in blood content which soon reached its maximum. The intake of 3,000 mg of nicotinic acid over a period of 15 days left this maximum unaffected.

3 Corresponding to the rise of nicotinic acid content of blood there was a sharp increase in the urinary output of nicotinic acid and a peak was reached in the first hour.

4 In fasting subject the nicotinic acid excretion in urine was lowered, but the response to a 'test dose' was enhanced.

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DARK-ADAPTATION TESTS IN CASES OF CLINICAL NIGHT-BLINDNESS

BY

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THE association between vitamin A deficiency and night-blindness has long been recognized. Visual purple, a photo-sensitive pigment in the retina of the eye, is concerned in the adaptation of the normal eye to dim light. The relation between vitamin A and the regeneration of visual purple was demonstrated by the experiments of Fridericia and Holm (1925) and Tansley (1931). The recent researches of Wald (1933, 1934, 1935, 1936) make it clear that the pigment is a conjugated protein with vitamin A, and the latter is a precursor of visual purple. It has been shown by Wald (*loc cit*) that light converts visual purple in the isolated retina to visual yellow which in turn is partly re-converted to visual purple and in part decomposed to some colourless products. In the living animal, visual purple is re-synthesized and decomposed repeatedly during light adaptation. It would appear that a large amount of vitamin A is normally present in the pigment epithelium of the retina and is essential for the regeneration of visual purple. Recent work on the part of Granit, Munsterhjelm and Zewi (1939) suggests the physiological reactions underlying dark adaptation are less simple than Wald's findings suggest.

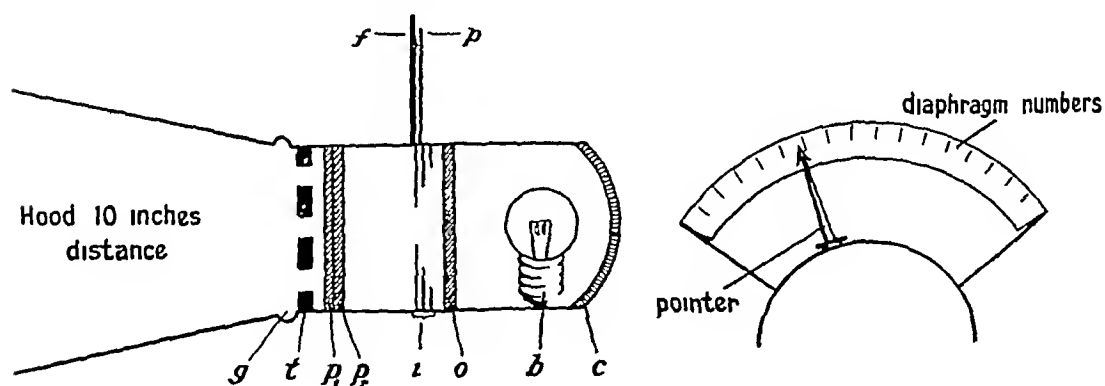
In an individual subsisting on a vitamin A deficient diet, the amount of vitamin A reaching the retina may be insufficient for its sensitization for vision in dim light through the medium of visual purple. Such an individual may be expected to show a slower rate of adaptation than a normal subject. This idea has been made the basis of tests for assessing degrees of vitamin A deficiency by the study of dark adaptation.

Few workers in this field have had the opportunity of studying the dark-adaptation curves of cases of clinical night-blindness before and after administration of vitamin A. By clinical night-blindness or hemeralopia is meant a condition in which vision in dim light is so impaired as to cause serious inconvenience and impel the patient to apply for medical treatment. In the present investigation 10 such cases

have been studied and for purposes of comparison the dark-adaptation curves of 20 normal individuals have been determined. The study of outspoken cases throws light on the relation between vitamin A and the course of adaptation and on the validity of dark-adaptation tests in the detection of vitamin A deficiency.

A number of instruments are in use for carrying out dark-adaptation tests, and they are based on the following principle. Under certain standard conditions the photo-sensitive pigment is first bleached by exposure to a powerful source of light and the regeneration of visual purple in the dark is tested by judging the individual's ability to discern a faintly illuminated test object. Within recent years a great deal of work has been carried out on the test and conflicting results have been reported. The recent papers of Thomson *et al* (1939) and Harris and Abbasy (1939) provide excellent reviews of the extensive literature on this subject.

Apparatus used—An 'adaptometer' of the Birch-Hirschfeld type, constructed in the Laboratory, was used. A sectional diagram is shown in the Text-figure. The apparatus is enclosed in a blackened metal casing. *b* is a microscope lamp of 3.45 c.p. (determined with a Lummer-Brodhun photometer) working at 6 volts (current consumption 1.2 amperes). A variable resistance is used to ensure a steady supply of current throughout the period of testing. *c* is a concave mirror reflecting the light from the bulb in parallel rays. *o* is a circular opal plate in front of the bulb which diffuses the light. Immediately in front of the circular opal plate a metal iris-diaphragm, taken from a good Zeiss microscope, is introduced.



TEXT FIGURE

- | | |
|------------------------------------|---|
| <i>c</i> Concave mirror | <i>f</i> Fan shaped enlargement (scale marked) |
| <i>b</i> Electric bulb (3.45 c.p.) | <i>p</i> ₁ and <i>p</i> ₂ Thick opal plates |
| <i>o</i> Opal plate | <i>t</i> Test object (Quincunx) |
| <i>i</i> Iris-diaphragm | <i>g</i> Groove to take rack carrying glass filters |
| <i>p</i> Pointer on scale | |

The small handle of the iris-diaphragm is soldered to a narrow long pointer. There is a narrow transverse slot on the upper segment of the apparatus at the position of the iris-diaphragm, allowing movement of the pointer from outside the metal case. Near the diaphragm, the apparatus is expanded in the shape of a fan,

the edge of which is marked in figures on the side facing the lamp. When the pointer is moved in the slot, its tip traverses the scale, indicating the diameter of the aperture of the diaphragm. A little in front of the iris-diaphragm, two thick opal plates in apposition to each other are introduced into the metal case. Close to and in front of these plates a black piece of cardboard with five holes, punched out like the marking on a dice, is introduced. The diameter of a hole is 4 mm and the five holes together cover an area of 15×15 sq cm. If the light is switched on in the apparatus, one can see five spots of light at the surface. A hood, 10 inches long (reading distance), of blackened cardboard facilitates observation of the test object. Close to the latter, the metal case has two horizontal grooves, along which a cardboard carrier containing neutral glass-filters (Tscherning's photometric glasses) of 10^{-1} , 10^{-2} , 10^{-3} , and 10^{-4} powers of transmission, can be slid in order to diminish further the intensity of light of the five spots.

To determine the proportion of light passing through the iris diaphragm at any required aperture, the following method was adopted. The aperture of the diaphragm at a certain opening was noted and the five spots of light (the test object) for this opening were photographed on a special rapid photographic plate. The test object, at various known values of opening of the diaphragm, was photographed on the same photographic plate. The densities of these images were then compared in a microphotometer. (The author is greatly indebted to Dr C S Venkateswaran of the Physics Department, Indian Institute of Science, Bangalore, for assistance in this connection). With the data obtained a reference curve was established showing the relationship between the proportion of light passing through the diaphragm and the corresponding opening of the diaphragm. This photographic method of recording the amount of light passing was adopted in order to eliminate any possible error due to imperfection of the diaphragm. It was, however, subsequently found that the curve obtained in this manner was essentially the same as that obtained in the usual manner by applying the law of inverse squares.

The 'bleaching' apparatus used resembled that described by Thomson *et al* (*loc cit*). It is a hemispherical bowl 2 feet in diameter and 18 inches deep. At its upper margin a 200 watt electric bulb is fixed to illuminate the white inside surface. Illumination from such a surface, as shown by Thomson *et al* (*loc cit*), more effectively bleaches the visual purple over a large uniform area in the retina than when a small or large illuminated plane surface is employed, as in some methods.

Procedure—The test is carried out in a dark-room. The bleaching apparatus and the adaptometer are placed side by side on the same table and the height of the apparatus suitably adjusted. The nature of the test is explained to the subject and a short 2 minutes' demonstration given to gain his co-operation. Then with the subject comfortably seated on the stool facing the bleaching bowl, and with his eyes at a level of the centre of the hemisphere, the light in the bowl is switched on. The sudden appearance of very bright light causes slight discomfort in some subjects, this is overcome by asking them to blink rapidly for a moment. The subject gazes steadily at the bright surface for exactly 4 minutes when the light is switched off. He then gets on to the adjacent stool and looks through the hood of the

adaptometer The light in the adaptometer is switched on The cardboard rack containing the neutral light filters is ready in the groove in front of the Quincunx so that the desired filter can be slid into position quickly opposite the test object This is accomplished by feeling the notch cut on the cardboard carrier to indicate the position of the light filter After the desired filter is slid into position, the pointer attached to the iris-diaphragm is moved from position zero up the scale until the subject says that the test object is very faintly visible The light is now switched off By means of a small pocket torch a small spot of light is allowed to fall on the stop-watch and the time that has elapsed between the extinction of the bright light and the response of the subject noted As the markings on the apparatus are towards the side facing the source of light no extraneous light reaches the subject As an additional precaution, the subject is asked to keep his eyes shut while readings are being recorded Often it is possible to take the first reading 20 to 25 seconds after the extinction of the bright light But the readings taken within $1\frac{1}{2}$ to 2 minutes are not very trustworthy, because the threshold for light for an eye in the course of adaptation falls very rapidly in the initial stages and is difficult to time accurately

A series of readings are taken at known intervals for a period of 10 minutes and thereafter at 5-minute intervals until the eye is completely dark-adapted and no lower threshold can be reached In normal subjects it is necessary to use higher grades of neutral glass-filters as the time of adaptation in the dark increases There are two controls for altering the brightness of the test object, viz (i) the neutral filter in front of the Quincunx and (ii) a finer adjustment by the movement of the pointer attached to the iris-diaphragm The veracity of the answers given by the subject is tested from time to time by suddenly cutting off the light without his knowledge and sometimes the pointer is moved to a much smaller value and his answers checked When the final visual threshold is reached, as indicated by the pointer being at a certain value on the scale with the proper grade of neutral light filter in position, it is evident that the subject will be unable to see any dimmer light by either narrowing the aperture of the diaphragm, keeping the light filter in position, or by introducing a higher grade of filter, keeping the position of the pointer the same This final visual threshold reading is checked again after 5 and 10 minutes Usually readings are taken up to 45 minutes

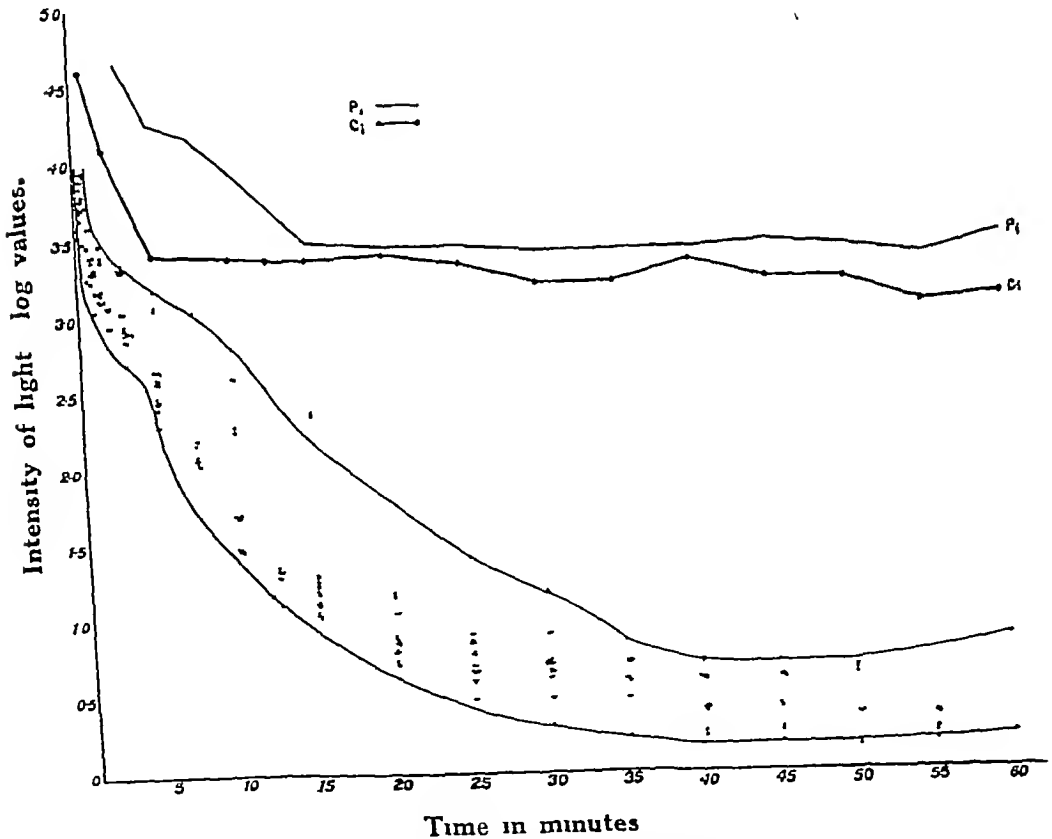
In recording values the following points were taken into consideration The lowest intensity of light visible to completely dark-adapted eyes in normal subjects can be recorded by using a neutral glass-filter of 10^{-4} transmission—when this grade of filter is used the pointer has to be moved in the neighbourhood of the 6 mm diameter of the aperture of the diaphragm If at the beginning of dark adaptation no light filter was needed and the intensity of light was controlled only by the pointer and for recording the final visual threshold, a filter of 10^{-4} transmission was found necessary, it follows that the intensity of light was diminished by $1/10,000$ of the original value This $1/10,000$ of light intensity is called '1' The light threshold at various intervals of time during dark adaptation is expressed in terms of this unit and comparative results for different subjects are thus obtained

The logarithm of these figures and the corresponding period in the course of dark adaptation are plotted and the dark-adaptation values expressed graphically

Results—Graph 1 shows the dark-adaptation readings of 20 normal subjects

The subjects were fairly well-nourished young laboratory workers and assistants and under the conditions of the test the final visual threshold was reached

GRAPH 1



The dark-adaptation curves of 20 normal subjects

Scattered points bound by two curves—normal subjects. The two curves P_1 and C_1 are for two sufferers from night-blindness

in 30 to 45 minutes after which no further change took place. In 2 cases giving values falling within the normal range, 216,000 I U of vitamin A (in the form of 'Prepalm') were given without appreciably influencing the course of adaptation. There was some variation in the values obtained for the same

person on different days and in those observed in different normal subjects. A reasonable range for normal values can, however, be given. This is shown in Graph 1 and can be used as a standard of comparison with the curves given by night-blind subjects. The graph includes two curves given by 2 such subjects (curves P_1 and C_1).

Cases of night-blindness —The investigation of 10 cases of night-blindness was carried out in two ophthalmic clinics at Coimbatore. As far as possible other causes of night-blindness were excluded by examinations carried out by the ophthalmologists in the respective clinics. The subjects were malnourished individuals living on a diet consisting mainly of rice with no milk, eggs or meat and leafy vegetables in negligible quantity. Seven of the 10 showed xerophthalmia and phrynoderma, and stomatitis were present in 2 of these cases. Stomatitis was observed in one case not showing xerophthalmia. The other two cases showed no visible evidence of vitamin deficiency apart from night-blindness. Except in one instance, the patients gave a history of having suffered from night-blindness for a month or more.

The response of dark adaptation to treatment with vitamin A in a typical case is illustrated in Graph 2. A typical normal curve is included for comparison. Curve (i) depicts the course of dark adaptation before the administration of vitamin A. The test was carried out in the morning, an hour later, the patient was given 3 c.c. of vitamin A concentrate orally ('Prepalin') equivalent to 216,000 I.U. in a single dose. Curve (ii) represents the values obtained 5 hours after dosing. Forty-eight hours later the subject himself volunteered the information that from the evening the dose was administered, he was able to see well in the dark.

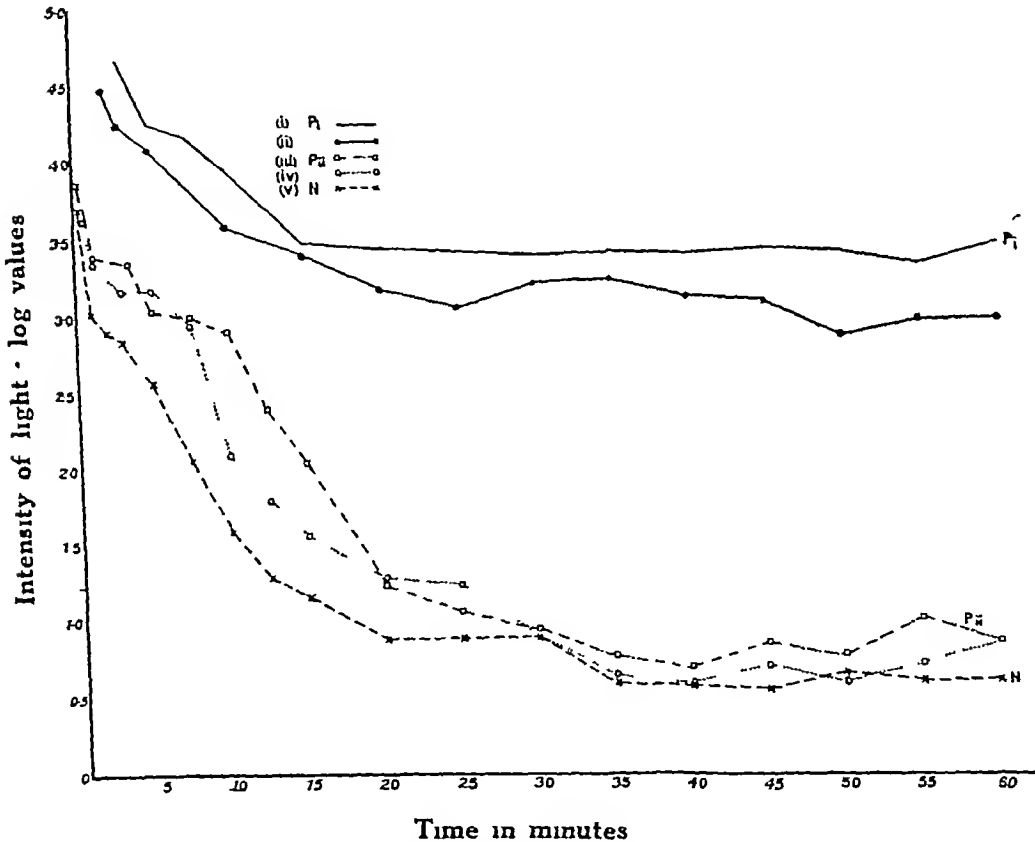
It can probably be assumed that the improvement shown by curve (ii) (5 to 6 hours after the experimental dose) was real. The test carried out 48 hours after the administration of vitamin A concentrate is shown in curve (iii). This curve falls within the normal range. The final visual threshold in this case showed a tendency to rise from the fourth day after treatment. On the fifth day a second dose, equivalent to 144,000 I.U., was given orally. The curve for dark adaptation obtained the next day is shown as curve (iv). It overlaps a typical normal curve (v). The sensitivity of the dark-adapted retina in this case and other similar cases was increased about 200 times by treatment with vitamin A, a change equivalent to 2.5 units on the logarithmic scale.

Excepting for 'R' [curve (i)] all were given 216,000 I.U. of vitamin A orally and in each case normal values were obtained within 24 to 48 hours.

The curves given by patient 'R' are of special interest. Previous to testing he had suffered from night-blindness for only a week, the condition being less severe than in the other subjects. A concentrate made from shark-liver oil produced in Malabar was administered. About 60 grammes of the oil, which was rich in vitamin A, were saponified. The yield of non-saponifiable matter was about 2.5 g. This was suitably diluted in a small quantity of arachis oil to obtain a clear oily solution. Spectrographic assay of the oil showed that the diluted oil had the potency of 146,000 I.U. per c.c. The patient was given 1.5 c.c. (approximately

equivalent to 219,000 I U of vitamin A) orally Improvement occurred in about 6 hours after dosage and 24 hours later the patient volunteered the information that he was able to see well the previous evening The test carried out 24 hours

GRAPH 2



Dark-adaptation curves of a typical case of night blindness before and after treatment

- (i) Curve before administering vitamin A
- (ii) Five hours after administering 216,000 I U of vitamin A
- (iii) Forty-eight hours later
- (iv) Twenty four hours after a second dose of 144,000 I U
- (v) A typical normal curve for comparison

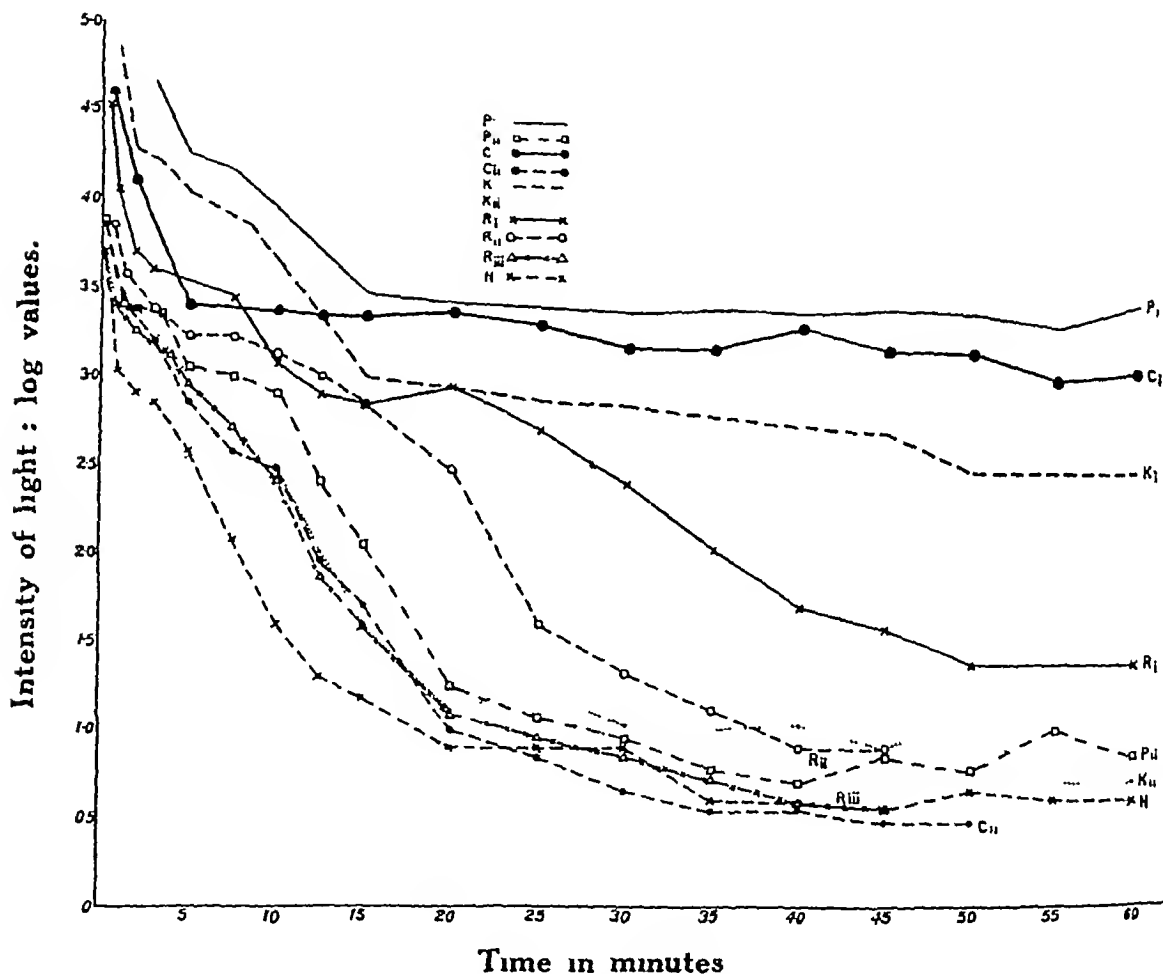
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after dosing certainly showed that the subject had recovered (curve R_{III}) It is clear that vitamin A concentrates made in India from shark-liver oil could replace many proprietary vitamin A concentrates at present imported. The present use of shark-liver oil as a substitute for cod-liver oil is a good beginning and there should

be scope for developing this industry so as to include the production of vitamin A concentrates

The group of 10 cases included 6 which had been given cod-liver oil for a period of a week or 10 days (about 3,500 I U of vitamin A daily according to

GRAPH 3



Dark adaptation curves of 4 typical cases of night blindness before and after treatment

R_I , K_I , C_I , P_I = Curves obtained before treatment

R_{II} = Six hours after administration of 219,000 I U of vitamin A (shark liver oil conc)

R_{III} = Twenty-four hours later

C_{II} , K_{II} , P_{II} = Forty eight hours after administration of 216,000 I U of vitamin A

N = A typical normal curve for comparison

spectrographic tests on the oil in question carried out in the Laboratories) Some improvement had apparently taken place in dark adaptation in these subjects, which was shown by a slight fall in the curve towards the normal range. A single dose

of 216,000 I U of vitamin A restored the curves to normal. These observations suggest that high dosage with vitamin A is necessary to obtain dramatic results in clinical night-blindness.

DISCUSSION

Typical untreated cases of night-blindness exhibited a greatly diminished rate of dark adaptation and the visual threshold reached after 60 minutes' dark adaptation was very much higher than in normal subjects. In the cases partially treated with cod-liver oil the rate of adaptation was not as greatly diminished as in typical untreated cases. The final visual threshold was higher than normal.

Edmund and Clemmesen (1936) recorded improvement in cases of hemeralopia in 7 to 10 minutes following a parenteral injection of 20,000 I U of vitamin A. Wald, Jeghers and Aramio (1938) observed a response in 30 minutes after administering orally 100,000 I U, whereas Hecht and Mandelbaum (1939) found that 100,000 I U of vitamin A by injection produced a transient improvement and that 50,000 I U daily in addition to the ordinary dietary for a period of a month were required to restore the dark-adaptation curve to normal. Results obtained in the present investigation are not completely in line with either of these findings. The present work definitely shows that there is improvement within 48 hours when very large doses of vitamin A are given. Discrepancies in the findings of different workers might be partly due to the different degree of impairment of vision in the cases studied.

Aykroyd (1930) observed clinical improvement in night-blindness occurring in Newfoundland fishermen 24 hours after the administration of 1½ oz. of crude cod-liver oil (equivalent to about 60,000 I U of vitamin A). It is possible that in Aykroyd's cases the dark-adaptation curves did not return completely to normal but sufficient improvement took place to remove the social inconveniences of night-blindness and satisfy the patients. The present investigation confirms the observation that in clinical night-blindness improvement in dark adaptation may take place rapidly, i.e. within 24 hours, on treatment with vitamin A.

Possibly the large dosage of vitamin A concentrate administered to the patients was unnecessary and equally good results might have been obtained with a smaller dose.

There are certain drawbacks to the routine use of dark-adaptation tests. They cannot be applied to young children or mentally dull people who cannot understand the test. In the present investigation no subject below the age of 11 years was studied. It is possible that widely different age groups may have different normal rates of dark adaptation. In the present study the 4 previously untreated cases of night-blindness and the majority of the normal subjects were in the age group 20 to 30 years. The long time taken by the test, the tedium of sitting for this period in the dark and the necessity of repeating the tests in some cases, are further disadvantages. It may be possible by devising a test of short duration to detect in about 10 to 15 minutes a change in the rate of dark adaptation in marked cases of vitamin A deficiency (as in clinical night-blindness), but to detect mild

degrees of impairment in adaptation a test sufficiently prolonged to enable a stable visual threshold to be reached would be necessary. The present investigation suggests that in pronounced cases of clinical night-blindness there is a clear-cut relationship between vitamin A and dark adaptation. Whether the same relation holds good in subjects showing minor degrees of variation in adaptation closer to the normal range is a matter for further investigation.

SUMMARY

1 The dark-adaptation curves of 10 cases of clinical night-blindness and 20 normal subjects were determined by an adaptometer of the Birch-Hirschfeld type. In the former there was a slower rate of adaptation and a higher final visual threshold.

2 Treatment with a large dose of vitamin A (216,000 I U) produced improvement within 6 hours and within 48 hours the curves returned to the normal range.

ACKNOWLEDGMENTS

The help of Dr A Sathianadhan, Ophthalmic Department, Government General Hospital, Coimbatore, and Drs Gurubatham and Roberts, Swedish Mission Eye Hospital, Coimbatore, is gratefully acknowledged. The 'Prepalin' used in the investigation was kindly supplied gratis by H J Foster & Co, Ltd, Bombay (representatives of Glaxo Laboratories). Thanks are due to Lieutenant A M Thomson, R A M C, formerly a worker in the Rowett Institute, Aberdeen, who visited the Laboratories and offered helpful criticisms.

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INVESTIGATIONS INTO THE EPIDEMIOLOGY OF EPIDEMIC DROPSY

Part XII

ISOLATION OF ACTIVE SUBSTANCES FROM TOXIC OILS *

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It has been shown in a previous communication (Lal, Mukherji, Das Gupta and Chatterji, 1940) that the reacting substance which has been isolated in pure form serves to indicate the amount of argemone oil present in mustard oil. Suggestive evidence has also been obtained that the degree of toxicity of oil corresponds roughly with its reacting substance content. A study of the physical, chemical and biological properties of this substance was undertaken in the hope that it might throw some light on the nature of the toxic substance.

A METHOD OF ISOLATION

The reacting substance (white crystalline substance) can be obtained in fair amounts by a simple process

* Read at the Indian Science Congress, January 1941

One hundred c c of oil, 25 c c to 30 c c of absolute alcohol and 25 c c to 30 c c of saturated solution of caustic potash are vigorously shaken together. To accelerate the process of saponification the mixture is heated to just below 100°C for half to one hour. It is then allowed to cool. Equal amount of distilled water is added and the emulsion is left undisturbed for a few days. Small shining mica-like crystals soon appear which separate out on further dilution with distilled water and are slowly deposited at the bottom of the vessel. The rate of crystal formation increases if the mixture is kept in the refrigerator.

To obtain the crystals the emulsion is filtered through a filter-paper. The crystals are repeatedly washed with distilled water to remove the soap. Being soluble in hot alcohol and only partially soluble in cold, they are purified by dissolving in hot alcohol and re-crystallizing by cooling. The substance is then dried in hot air-oven at 50°C to 60°C and finally under vacuum at 100°C until constant weight is maintained.

B NATURE AND PROPERTIES

1 *Physical properties*

(i) *General*—The crystals are white, shining like bits of mica, under the microscope they give the appearance of thin rhomboidal plates mostly occurring in bundles (*vide* Plate X).

The substance has a sharp and constant melting point between 188°C and 189°C. This fact indicates its purity.

The density determined by the flotation method using potassium iodide solution for the purpose is 1.42 at 30°C.

In alcoholic solution the substance exhibits a strong bluish-violet fluorescence. The absorption spectrophotograph exhibits two broad absorption bands. One of them is between 3,120 Å U and 2,560 Å U with the maximum at 2,760 Å U and the other band between 3,120 Å U and 3,400 Å U with a maximum at 3,240 Å U.

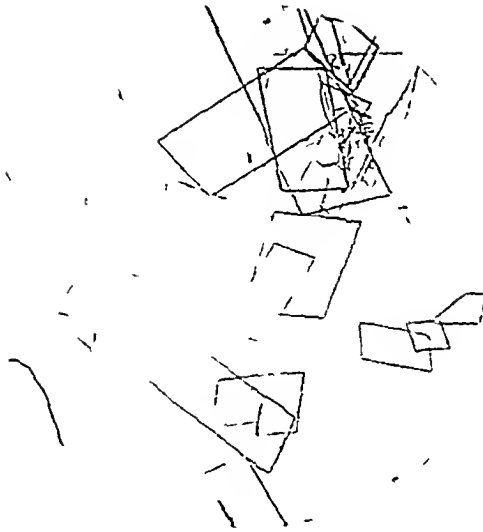
(ii) *Solubility*—The substance is insoluble in both cold and hot water, sparingly soluble in cold absolute alcohol but more so in hot alcohol. It is readily soluble in most of the ordinary organic solvents, e.g. chloroform, acetone, carbon tetrachloride, carbon disulphide, benzene, ether, toluene and hot amyl alcohol. Dissolved in chloroform and carbon tetrachloride it develops a slight yellow colour which in the former solvent deepens on exposure to light.

It is insoluble in strong or dilute alkali even when heated but it is soluble in dilute hydrochloric acid, more particularly on warming.

2 *Chemical properties*

(i) *Colour reaction*—The crystals give characteristic colour reactions with certain reagents (*vide* Table). For purposes of comparison corresponding reactions given by berberine and protopine are also noted (Mitchell, 1929, Small, 1932).

PLATE X



Microphotograph of the 'white crystalline substance' $\times 400$

TABLE

Chemical reactions of the white crystalline substance as compared with those of berberine and protopine

Reagents	Reacting substance (white crystalline)	Berberine	Protopine
Conc. HNO_3	Reddish brown	Reddish brown	
Conc. H_2SO_4	Yellow turning to amber colour and on heating greenish black	Orange yellow changes to olive green on warming	Yellow turning to blue violet, then green
$\text{K}_2\text{Cr}_2\text{O}_7$ in conc. H_2SO_4	Deep-brown	Black changing to violet	Intense blue
Mixture of CH_3COOH and H_2SO_4	Faint-yellow	Deep-yellow	Deep-blue violet
Froehde's reagent	Yellow, olive and finally orange red.	Immediate yellow change through dark brown to violet brown.	Olive turning violet, then green
Solution of cupric acetate in glacial acetic acid	On heating the blue colour of the solution changes to green.	On heating the blue colour of the cupric acetate solution changes to deep-green	

(ii) *Alkaloidal reactions*.—Dissolved in dilute hydrochloric acid the substance gave the following reactions —

- | | | |
|---|--|--|
| 1 | Mayer's reagent | White precipitate |
| 2 | Saturated solution of picric acid in water | Deep-yellow shining precipitate |
| 3 | Conneschein's reagent | Brownish yellow precipitate, supernatant solution bluish green |
| 4 | Tannic acid solution in water | White precipitate |
| 5 | Saturated solution of mercuric chloride in water | White precipitate |
| 6 | Wagner's reagent | Heavy-chocolate precipitate |
| 7 | Mayer's reagent | Colour changed from white to pale yellow |
| 8 | Platinic chloride solution in water | Faint brown coloured crystalline precipitate |

These results suggest that the reacting substance is alkaloidal in nature

3. Chemical composition

The results of microchemical analysis (two consecutive readings) are as follows —

Percentage of C and H determined by Pregl's method—

$$\text{C} = 71.40 \quad 71.30$$

$$\text{H} = 4.35 \quad 4.38$$

Percentage of N determined by Micro-Dumas's method—

$$\text{N} = 4.37, \quad 4.50$$

The empirical formula of the substance is therefore $\text{C}_{15}\text{H}_{15}\text{O}_2\text{N}$

4 Molecular weight

The molecular weight was determined by combining the base with platinum chloride. It was found to be 326. This figure closely approximates to the one obtained from the empirical formula, viz 321.

In the preparation of the reacting substance, the process of saponification involved a rather drastic treatment and it was thought that this circumstance might damage or denature the toxic substance present in the toxic oil in the native form. Using another method to meet this objection, a crystalline free base was obtained in pure form (to be called C F B for short).

Crystalline free base (C F B) —

(a) *Method of isolation*—Hydrochloric acid gas is passed through argemone oil dissolved in equal volume of dry ether, till precipitation is completed. The precipitate is separated by rapid filtration through a Buchner funnel in vacuum. It is repeatedly washed with dry ether to remove the last traces of oil. It is then dried in vacuum desiccator. To obtain the free base the substance is washed in 33 per cent liquor ammonia or repeatedly in hot water.

The substance is purified by dissolving in minimum quantity of hot toluene and precipitating by the addition of equal volume of hot absolute alcohol. If the process is repeated a number of times the compound is obtained in pure form. It is dried in vacuum at 100°C.

(b) *Nature and properties* *Physical properties*—(i) General—It is a snow-white crystalline compound and looks like common salt. It has a sharp melting point at 190°C. Solution in alcohol exhibits a strong bluish-violet fluorescence. (ii) Solubility—This is same as of the reacting substance.

(c) *Chemical properties*—Colour reactions and alkaloidal reactions are also the same as of the reacting substance.

(d) *Chemical composition*—The microanalytical data are as follows (two consecutive readings) —

Carbon	72.06, 71.94
Hydrogen	4.56, 4.48
Nitrogen	4.44, 4.46

The empirical formula works out to be $C_{19}H_{15}O_4N$ which is identical with that of the reacting substance. Thus, the two substances though rather different in their physical appearance and obtained by two different methods are chemically almost identical.

DISCUSSION

The biological properties of the reacting substance and of the free base will be described in a separate communication. However, it may be stated here that both of them contain a radical which is responsible for the differential tests of toxic oils described in a previous communication (Lal, Mukherji, Roy and Sankaran, 1939). It may also be mentioned that argemone oil which has been exposed to

light for a sufficiently long period to greatly reduce its reactivity to the differential tests (Lal *et al* , 1940) yields a very poor crop of the reacting substance or the free base. As will be shown in a subsequent paper, argemone oil which has almost lost its reactivity to the tests due to either exposure to light or to extraction of the crystalline free base (C F B) also loses its toxic properties. From these results it would appear that the reacting substance or the crystalline free base represents a part of the complex molecule of the specific toxin as found in the oil. This compound is liable to break up by a relatively simple chemical treatment.

SUMMARY

1 Two methods of isolation of substances which are responsible for the reactivity of the argemone oil (or mustard oil epidemiologically associated with epidemic dropsy) have been described.

2 Reasons are given to suggest that these substances represent part of the complex molecule of the native toxic substance in the oil.

ACKNOWLEDGMENTS

We are glad to express our gratefulness to Professor P C Mitra, M A , Ph D , Paht Professor of Chemistry, for his guidance and supervision in connection with microanalytical work, and for the facilities provided by him for the purpose. We also take pleasure in acknowledging the valuable assistance given by Mr N Ghosh, M Sc , of Professor Mitra's department, in repeating the results of microchemical analysis.

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SEPARATION OF THE NEUROTOXIN FROM THE CRUDE
COBRA VENOM AND STUDY OF THE ACTION
OF A NUMBER OF REDUCING
AGENTS ON IT

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It has been reported by us in a previous publication (Ghosh and De, 1937) that starting with a sample of dry cobra venom the pigeon unit of which was 0.3 mg (one pigeon unit being defined by us as that quantity of venom which, when injected intramuscularly, was just sufficient to kill a pigeon weighing 300 g to 310 g), the neurotoxin was separated from the hæmolysin and was concentrated to such an extent that its pigeon unit was found to be 0.018 mg (dry weight). While examining the toxicity of samples of cobra (*Naja tripudiana*) venom procured from different sources, a sample of venom from the Drug House (India), Ltd., was found to be much more toxic than the others. Having been assured of a steady supply of this quality of highly toxic venom, attempt was made to isolate the neurotoxin from it. The results we have obtained so far are recorded in this paper.

CONCENTRATION OF NEUROTOXIN

1 *Precipitation of the impurities with sodium sulphate*—The crude cobra venom contains, besides the neurotoxin, a number of enzymes, and a considerable

proportion of inactive protein. It was found by experiment that the major portion of these latter substances could be removed by fractional precipitation with sodium sulphate. A stock solution of sodium sulphate was prepared for this purpose by using 44 g of anhydrous sodium sulphate per 100 c c of water at 37°C and the precipitation was carried out in two steps (a) and (b) using different concentrations of the electrolyte. In the first step (a), to 100 c c of a 0.5 per cent solution of the cobra venom was added with constant stirring an equal volume of the sodium sulphate solution and the mixture left in a thermostat adjusted at 37°C for 30 minutes. It was then filtered and the precipitate dissolved in 50 c c of water and re-precipitated with the addition of 50 c c of the sodium sulphate solution. This process was repeated once more and the different filtrates were combined. The precipitate was found to contain about 18 per cent of the neurotoxin, while the combined filtrate contained more than 80 per cent of the neurotoxin. The next step (b) consisted of further fractionation of this combined filtrate and it was carried out in the following way. The volume of the filtrate was measured and for every 100 c c of the filtrate 48 c c of the sodium sulphate solution were added with constant stirring. The mixture was left in a thermostat at 37°C for 30 minutes and then filtered. The precipitate was dissolved in 50 c c of water and then treated with 100 c c of the sodium sulphate solution. After incubation at 35°C for 30 minutes, it was filtered. The filtrates were combined and it was found to contain about 55 per cent neurotoxin and 12 per cent protein.

2 *Precipitation of the neurotoxin with tungstic acid*—The volume of the combined filtrate was measured and for every 30 c c of the filtrate, 1 c c of 2/3 N sulphuric acid and 1 c c of a 10 per cent solution of sodium tungstate were added with constant stirring. The mixture was left at room temperature for about 10 to 15 minutes and then centrifuged. The supernatant liquid was decanted off and the precipitate washed twice by centrifugation using each time 10 c c of an aqueous solution of sodium tungstate and sulphuric acid in the same proportion in which they were used before. After the washing was completed, the precipitate was suspended in 10 c c of water and N/5 sodium hydroxide solution was added to it drop by drop with repeated stirring until the solution became clear and alkaline (pH 8.6 to 8.8). To this solution was then added barium chloride in slight excess to remove the tungstic acid as insoluble barium tungstate. The mixture was centrifuged and the supernatant liquid containing the neurotoxin was withdrawn. It was neutralized with dilute sulphuric acid solution and any barium ions contained in it was precipitated by treatment with just sufficient quantity of dilute sodium sulphate solution. It was filtered, the filtrate cooled in an ice-chest and then treated with four times its volume of ice-cold methyl alcohol, when a precipitate was formed containing the neurotoxin. The mixture was centrifuged, the supernatant liquid was decanted off and the precipitate dried in a vacuum desiccator. The dry substance thus obtained will be called neurotoxin (1). The toxicity and protein content of the neurotoxin (1) was determined and it was found that 1 mg of its protein was associated with 105 pigeon units.

3 *Fractional precipitation with methyl alcohol*—The neurotoxin (1) prepared in the way described above was dissolved in water cooled to about 2°C and subjected to fractional precipitation at different pH with ice-cold methyl alcohol. The experiments were carried out at a low temperature in order to avoid destruction of the neurotoxin owing to denaturation. The results are recorded in Table I. It will be noticed from the data that the optimum condition of precipitation is near about pH 2.8 using a volume of alcohol twice that of the aqueous solution. The precipitate obtained under the above optimum condition was washed several times by centrifugation using a mixture of methyl alcohol and water in the proportion of two volumes of alcohol to one volume of water. Finally, it was washed once with absolute methyl alcohol and dried inside a vacuum desiccator. This dried substance will be referred to as neurotoxin (2). One pigeon unit of neurotoxin (2) contained only 0.008 mg of protein. The procedure described above was repeated many times and was found to yield consistent results.

TABLE I

Volume in c.c. of 0.034 per cent neurotoxin (1) solution used	pH adjusted at	Volume in c.c. of methyl alcohol added	Weight in mg of protein precipitated	Number of pigeon units of neurotoxin associated with the precipitate
5	2.2	10	1.08	143
5	2.8	10	1.50	188
5	5.0	10	1.22	145
5	7.0	10	1.10	125

4 *Fractional precipitation with ammonium sulphate*—Besides methyl alcohol, ammonium sulphate was also used in an attempt to concentrate the neurotoxin still further by fractional precipitation from a solution of neurotoxin (1). Fifty mg of the neurotoxin (1) were dissolved in 10 c.c. of water, the solution cooled to 6°C and 3 g of ammonium sulphate were added to it gradually while stirring the mixture. After 30 minutes it was filtered under suction using Whatman filter-paper No. 50, the filtrate cooled and again treated with 3 g of ammonium sulphate. After 30

minutes it was again filtered in the manner described above. The toxicity and the amount of protein in the different fractions are recorded in Table II —

TABLE II

Ammonium sulphate added in g	QUANTITY OF PROTEIN IN MG FOUND IN THE		Number of pigeon units of neurotoxin found in the precipitate	Number of pigeon units of neurotoxin left in solution
	Precipitate	Solution		
0	0	50	0	5,250
3	3	47	140	1,810
6	32.6	14.4	2,650	2,360

It will be noticed from the data recorded in Table II that the last filtrate obtained after a total quantity of 6 g of ammonium sulphate has been added still contains a considerable amount of neurotoxin associated with a relatively small proportion of protein. The separation of the neurotoxin from this filtrate by tungstic acid, its elution from the tungstic acid surface and its precipitation and drying were carried out in exactly the same way as described under the heading (2). The toxicity and protein content of this preparation to be referred to as neurotoxin (2A), was determined and it was found that per pigeon unit it contained 0.0061 mg of protein. The mouse unit of this neurotoxin (2A) was also determined by intramuscular injection into mice each weighing between 18 g and 20 g. It was found that 1 mg of neurotoxin (2A) contained 45,500 mouse units, one mouse unit being defined as that quantity of neurotoxin which on intramuscular injection kills a mouse weighing between 18 g and 20 g divided by the actual weight in grammes of the mouse used, i.e. if 1 mg of neurotoxin is required to kill a mouse of 20 g then the mouse unit is $1/20$ or 0.05 mg.

5 *A comparison of the activity of the cobra neurotoxin separated by different authors*—Investigation on the isolation of the neurotoxin of the cobra venom has been carried out by Ganguly and Malkana (1936), Ganguly (1937) and by Wieland and Konz (1936), by Micheel *et al* (1937) and by Ghosh and De (1938). Micheel *et al* report that on electrolysis of a solution of *Naja tripudiana* venom in a multi-chambered cell a solution was obtained in one experiment which contained 0.03% of protein per mouse unit. On evaporation under reduced pressure, the toxicity of the solution diminished considerably and the solid obtained contained

one mouse unit per 0.53γ of protein. The neurotoxin (2A) which we have separated in the solid state contained one mouse unit per 0.022γ of protein and is therefore about 1.4 times more active than the solution prepared by Micheel *et al*. This preparation (2A) therefore represents the most active neurotoxin that has yet been isolated in the solid state.

6 *Action of chemicals on the neurotoxin of cobra (Naja tripudiana) venom* — The action of cysteine and sodium bisulphite on the neurotoxins of *Naja flava* and *Naja tripudiana* has been investigated by Micheel *et al* (*loc cit*) and on the neurotoxins of *Crotalus t. terrificus* and of *Bothrops Jararaca* by Slotta *et al* (1938). These authors have found that both the compounds have got destructive action on the neurotoxins of the venoms investigated, the action of sodium bisulphite being found stronger than that of cysteine. Micheel *et al* explain their results by assuming that a neurotoxin molecule contains a thiolactone ring and inactivation is produced by the sodium bisulphite attacking the thiolactone ring and breaking it up. Slotta *et al* on the other hand assume that the neurotoxins contain -S-S-linkage in their molecules and that the cysteine or the sodium bisulphite attacks the -S-S-linkage and breaks it thereby producing inactivation of the neurotoxins. We have investigated not only the action of cysteine and sodium bisulphite but also of a number of other compounds possessing marked reducing properties on the solutions of the neurotoxin (1) isolated by us as well as on that of crude cobra (*Naja tripudiana*) venom. The pH of the solutions in all cases except where HCl was used was adjusted at about 7.0 by adding the requisite volume of dilute sodium carbonate solution. The results are recorded in Tables III and IV —

TABLE III
Solution of neurotoxin (1)

Compound used	Quantity of compound taken	Weight in mg of neurotoxin (1) taken	Volume in c.c. of solution.	NUMBER OF UNITS OF TOXIN IN 10 C.C. OF SOLUTION		
				At the beginning	After 24 hours	Loss of toxicity in per cent
Sodium bisulphite	20 mg	1	10	105	5	95.2
Sodium sulphite	20 "	1	10	105	92	12.4
Cysteine	200 "	1	10	105	79	24.7
Ascorbic acid	200 "	1	10	105	75	28.6
Zn + HCl (N/10)	2 c.c.	1	10	105	65	38.1
HCl (N/10)	2 "	1	10	105	90	14.3
H ₂ S (passed for 15 minutes)		1	10	105	105	Nil

TABLE IV.

Solution of crude cobra venom.

Compound used	Quantity of compound taken	Weight in mg of crude venom	Volume in c c of solution	NUMBER OF UNITS OF TOXIN IN 10 c c OF SOLUTION		
				At the beginning	After 24 hours	Loss of toxicity in per cent
Sodium bisulphite	20 mg	10	10	100	8	92
Sodium sulphite	20 "	10	10	100	91	9
Cysteine	200 "	10	10	100	93	7
Ascorbic acid	200 "	10	10	100	95	5
Zn + HCl (N/10)	2 c c	10	10	100	62	38
HCl (N/10)	2 "	10	10	100	90	10

It will be noticed from the data recorded in Table III that sodium bisulphite has got the greatest destructive action on the cobra neurotoxin. Cysteine and ascorbic acid produce only 25 and 29 per cent destruction respectively even at concentrations 200 times higher than that of the neurotoxin (1). Treatment with zinc dust and N/10 hydrochloric acid produces about 38 per cent destruction. But since N/10 HCl alone produces about 14 per cent destruction, the effect due to nascent hydrogen is therefore only 24 per cent. If the destruction of the neurotoxin was due simply to the reduction of the -S-S-linkage to -SH as has been suggested by some authors, then it would be difficult to explain how, of all the reagents used, sodium bisulphite alone could produce destruction to the extent of 95 per cent, while the maximum inactivation caused by the others was only 30 per cent. It appears likely that sodium bisulphite has some specific action on the cobra neurotoxin. We have found that the neurotoxin (1) contains some carbohydrate and it is likely that the bisulphite reacts with the carbohydrate and thereby produces inactivation. A comparison of the results in Tables III and IV will also show that sodium sulphite, ascorbic acid and cysteine destroy the neurotoxin (1) to a greater extent than the neurotoxin in the crude venom. This is perhaps due to the fact that the crude venom contains, besides the neurotoxin, many other substances and these react with the reagents and partly neutralize them so that their effect on the neurotoxin in the crude venom becomes less pronounced.

SUMMARY

1 A very active sample of neurotoxin has been separated from the crude cobra (*Naja tripudiana*) venom by fractional precipitation of impurities with sodium sulphite, adsorption of the neurotoxin on tungstic acid, followed by its elution and further fractional precipitation from the eluted solution by treatment with ammonium sulphate. The pigeon and mouse units of this neurotoxin were 0.0061 mg and 0.022 γ respectively.

2 The action of a number of reducing agents on solutions of cobra neurotoxin has been tried *in vitro* and it has been found that sodium bisulphite, zinc and hydrochloric acid (N/10) ascorbic acid and cysteine can destroy the neurotoxin to a marked extent.

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HÆMATOLOGICAL STUDIES IN INDIANS

Part XIII

NORMAL INDIAN WOMEN IN CALCUTTA

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IN earlier papers on this series (Parts II and V) we reported the blood findings in Indian men living in Calcutta (Napier and Das Gupta, 1935, 1936). An opportunity arose to obtain blood specimens from a number of normal Indian women living in Calcutta. The analysis of our findings is reported in this paper.

This investigation was undertaken with the assistance of the staff of the Indian Research Fund Association's inquiry into the anæmia of pregnancy in Calcutta which was conducted successively by the two senior writers. Drs (Misses) S Pandit, S Dharamvir and J Dei, and Dr D N Mukherjee assisted in taking the blood and in doing some of the counts and estimations. These were all experienced workers and the work was done in the senior writer's laboratory at the Calcutta School of Tropical Medicine by, or under the direct supervision of, the junior writer.

The subjects—The vast majority of the subjects were single women, they included doctors, medical students, health visitors, school girls, volunteers from the Y W C A and older girls from the London Mission and Scottish Mission hostels. There were 134 women, aged from 14 to 38, the various age groups were not evenly distributed enough for division of the subjects into annual age groups, but, in order to see if age has much influence on the hæmatological findings, we have divided the subjects into three main age groups. The original intention was to divide them into quinquennial groups, but, as this left only very small groups at each end of the scale, we decided to include these in the top and bottom

age groups, respectively. There was only one subject aged 14 and only 6 over 30. Subsequently, we separated and re-analysed the age groups 15 years and 16 years, as there were 24 and 20, respectively, in these two groups. We have thus analysed the data separately for three major age groups and for the individual years 15 and 16, in regard to hæmoglobin, total red cells, reticulocytes, mean corpuscular volume (MCV), mean corpuscular hæmoglobin (MCH) and mean corpuscular hæmoglobin concentration (MCHC), total leucocyte count, eosinophil percentage, Arneeth count (weighted mean) and platelets.

TABLE I-a

Showing hæmoglobin percentages (100 per cent = 13.75 grammes) in different age groups

	Age group	Number of subjects in each group	Mean	S. D.
A	15	24	88.458	± 7.896
B	16	20	92.300	± 5.731
C	14-19	67	90.149	± 8.452
D	20-24	36	91.083	± 8.04
E	25-38	31	90.452	± 9.60

TABLE I-b

Showing red cells in thousands per c mm in different age groups

	Age group	Number of subjects in each group	Mean	S. D.
A	15	24	4582.08	± 507.77
B	16	20	4586.50	± 393.98
C	14-19	67	4566.27	± 448.91
D	20-24	36	4629.72	± 372.66
E	25-38	31	4714.35	± 360.15

TABLE I-c

Showing reticulocyte percentages in different age groups

	Age group	Number of subjects in each group	Mean	S D
A	15	24	0.37	± 0.30
B	16	18	0.26	± 0.17
C	14-19	63	0.39	± 0.57
D	20-24	34	0.49	± 0.32
E	25-38	31	0.38	± 0.32

TABLE I-d

Showing mean corpuscular volume (MCV) in cu μ in different age groups

	Age group	Number of subjects in each group	Mean	S D
A	15	24	84.15	± 9.36
B	16	20	86.13	± 6.89
C	14-19	67	85.91	± 8.41
D	20-24	36	86.99	± 7.56
E	25-38	31	85.66	± 6.91

TABLE I-e

Showing mean corpuscular hæmoglobin (MCH) in $\gamma\gamma$ in different age groups

Age group		Number of subjects in each group	Mean	S D
A	15	24	26.85	± 3.77
B	16	20	27.57	± 2.39
C	14-19	67	27.27	± 3.21
D	20-24	36	27.32	± 3.19
E	25-38	31	26.53	± 3.32

TABLE I-f

Showing mean corpuscular hæmoglobin concentration (MCHC) percentage in different age groups

Age group		Number of subjects in each group	Mean	S D
A	15	24	31.89	± 1.87
B	16	20	32.14	± 1.22
C	14-19	67	31.77	± 1.64
D	20-24	36	31.27	± 1.84
E	25-38	31	30.88	± 2.49
	25-38	30	31.15	$\pm 2.01^*$

* Note —In one subject the MCHC was 22.7 per cent, this is 5 per cent lower than the next lowest figure. If this reading is excluded the mean, etc. is as shown in this line.

TABLE I-g

Showing the leucocyte count per c mm in different age groups

Age group		Number of subjects in each group	Mean	S D
A	15	24	7152.50	± 1890.87
B	16	20	7918.00	± 1844.55
C	14-19	67	7512.63	± 1813.96
D	20-24	36	7118.89	± 1697.84
E	25-38	31	6430.97	± 1491.62

TABLE I-h

Showing the eosinophil percentage in different age groups

Age group		Number of subjects in each group	Mean	S D
A	15	23	5.68	± 4.92
B	16	20	5.35	± 4.37
C	14-19	63	5.28	± 4.41
D	20-24	35	5.67	± 4.08
E	25-38	30	4.07	± 3.68

TABLE I-2.

Showing the Arineth count (weighted mean) in different age groups

	Age group	Number of subjects in each group	Mean	S D
A	15	22	1 71	$\pm 0 24$
B	16	12	1 63	$\pm 0 18$
C	14-19	54	1 70	$\pm 0 23$
D	20-24	33	1 80	$\pm 0 28$
E	25-38	25	1 87	$\pm 0 18$

TABLE I-3

Showing the platelet count in thousands per c mm. in different age groups

	Age group	Number of subjects in each group	Mean	S D
A	15	15	627 53	$\pm 391 82$
B	16	10	688 80	$\pm 402 65$
C	14-19	46	678 70	$\pm 388 30$
D	20-24	29	510 41	$\pm 388 64$
E	25-38	26	718 38	$\pm 499 74$

Difference between the findings in different age groups—From the tables the difference between the means of the findings in various groups was calculated and the significance of these differences was investigated. A significance table (Table II-a) for the hæmoglobin is given. It will be seen that in no case was the difference statistically 'significant', though the difference between the 15-year and 16-year age groups was not far short of the 'significance' level.

TABLE II-a

'Significance' table for data in Table I-a hæmoglobin

Groups contrasted.	Difference between means, m_1 and m_2	Standard error (S.E.) of difference	$\frac{m_1 - m_2}{S.E.}$	Significance
A and B	3.842	2.059	1.87	Not significant
A and D	2.625	2.096	1.25	"
A and E	1.904	2.372	0.84	"
B and D	1.317	1.854	0.66	"
B and E	1.848	2.161	0.86	"
C and D	0.934	1.692	0.55	"
C and E	0.303	2.024	0.15	"
D and E	0.631	2.196	0.27	"

In the case of the total red cell counts, there is little difference between any of the means and these differences are not 'significant'.

The means of the reticulocyte percentages were only worked out to two points of decimals, but this fact will not affect any conclusions one might draw. The mean of the reticulocyte percentages for the 16-year age group appears to be very low, significantly lower than that of the 20-to-24-year age group but not than that of the highest age group. There are only 18 subjects in this former group and it is

* By 'significant' we mean that the probabilities are at least 20 to 1, that the difference between two means is not due to chance, this is indicated by the difference between the means being more than twice the standard error of the difference.

doubtful if any importance need be attached to this observation The data are given in Table II-c —

TABLE II-c

'Significance' table for data in Table I-c, reticulocytes

Groups contrasted	Difference in means	Standard error of difference	$\frac{m_1 - m_2}{S E}$	Significance
B—D	0.23	± 0.06	3.83	Significant
B—E	0.13	± 0.07	1.71	Not significant

The three corpuscular values which are shown in Tables I-d, I-e and I-f are very constant and in no case is the difference between any two groups anything like 'significant'. As far as the MCHC is concerned even the readings within the various groups are very constant as is shown by the very low standard deviations from the means.

The case of the leucocyte counts is different and the three main age groups show a progressive downward tendency in the leucocyte counts, the difference between the youngest and oldest age groups is 'significant', and the difference between the middle and the oldest groups is just short of the 'significance' level (Tables I-g and II-g) —

TABLE II-g

'Significance' table of data in Table I-g leucocyte count

Groups contrasted	Difference between means	S E of difference	$\frac{m_1 - m_2}{S E}$	Significance
A and B	765.50	564.89	1.36	Not significant
A and D	33.61	479.59	0.07	"
A and E	721.53	469.83	1.54	"
B and D	799.11	500.20	1.60	"
B and E	1487.03	491.83	3.02	Significant
C and D	393.74	359.43	1.10	Not significant
C and E	1081.66	347.68	3.11	Significant
D and E	687.92	389.66	1.77	Not significant

The eosinophil percentages are again very constant. The percentage is highest in the oldest age group, but this mean is not significantly different from that of any of the other groups.

In the Arneeth count (weighted mean) the three main age groups show a progressive increase with age. The means of the youngest main age group and the two single year groups are 'significant' different from the oldest group, but the differences between the means of groups C and D and groups D and E are not quite 'significant'.

TABLE II-2

'Significance' table of data in Table I-1 Arneeth count

Groups contrasted	Difference between means, $m_1 - m$	Standard error of difference	$\frac{m_1 - m_2}{S.E.}$	Significance
A and B	0.08	0.07	1.14	Not significant
A and D	0.09	0.07	1.29	"
A and F	0.16	0.06	2.67	Significant
B and D	0.17	0.07	2.43	"
B and E	0.24	0.06	4.00	"
C and D	0.10	0.06	1.67	Not significant
C and E	0.17	0.05	3.40	Significant
D and E	0.07	0.06	1.17	Not significant

This shift to the left of the Arneeth count in younger subjects is well recognized and does not call for any special comment.

In the platelet count, the age group 20-24 shows by far the lowest mean count, but the standard deviations are so wide that the means are not 'significantly' different from those of the group above or the group below.

The normality of the subjects examined—We next examined the data to see if any of the individuals should be considered as abnormal. We took the hæmoglobin first. In 6 cases the hæmoglobin fell outside the range, mean $\pm 2 \times$ standard deviation. In these cases the hæmoglobin was 65, 66, 68, 70, 71 and 72 per cent and in no other instance was it less than 77, so that there was a clear line between these 6 cases and the rest. The case for excluding these 6 subjects as anæmic, therefore, seemed a strong one. Examining the other data we found that the MCH was similarly outside the range ± 2 standard deviation in 5 of these 6 subjects. This was what one would expect, as the red cell counts were not markedly reduced in any case, but in 4 out of 6 the MCV was also outside this range, and, as this measurement is independent of the hæmoglobin, the observation provided additional

evidences that these subjects were abnormal. The subject with a low hæmoglobin but an MCV and MCH within the above range had a reticulocyte count of 4.4 per cent and an erythrocyte-sedimentation rate (ESR) of 54 in one hour.

In a few other instances a single measurement, other than that of the hæmoglobin, of other individual subjects fell outside the normal range, but, as in these it was only this one measurement, the exclusion of the individual concerned did not seem justified.

Having thus ensured the homogeneity of the material we re-calculated the mean and standard deviation for the whole 128 subjects. The figures are given in Table III —

TABLE III
*Showing findings in whole series after exclusion of 6
'sub-normal' subjects*

	Number of subjects	Mean	S. D.
Hæmoglobin percentage	128	91.49	± 7.31
„ grammes		or 12.58	± 1.01
Red corpuscles in thousands per c mm	128	4614.88	± 409.13
Reticulocyte percentage	122	0.37	± 0.27
Mean corpuscular volume	128	86.82	± 7.28
Mean corpuscular hæmoglobin	128	27.42	± 2.89
Mean corpuscular hæmoglobin percentage	128	31.57	± 1.76
Leucocytes per c mm	128	7161.77	± 1764.64
Eosinophils percentage	123	5.16	± 4.21
Arneth count (weighted mean)	108	1.77	± 0.25
Platelets in thousands per c mm	97	629.21	± 425.82

DISCUSSION.

There is not much matter for comment in Table III. The hæmoglobin percentage is a little lower than that of females of Bombay and Delhi and distinctly lower than the British and American figures, further, the sex difference is apparently greater in Calcutta than elsewhere. Our figure is nearly 20 per cent less than

a comparable figure for males in Calcutta, whereas the difference between the sexes according to Price-Jones (1931) is only 6·2 per cent, Whitby and Britton (1939) 12·2 per cent and Sokhey *et al* 15·5 per cent. On the other hand, the hæmoglobin mean is markedly higher than that of the tea-garden coolies (Napier, 1940). The mean of the red cell counts is practically the same as that of all female populations. The reticulocyte percentage was actually a little lower than we found in a similar series of men in Calcutta (Napier and Das Gupta, 1935). The corpuscular values are of the same order as those found by us (Napier and Das Gupta, 1936) and by other observers in normal populations. The white cell count comes a little higher than that of Calcutta men, but lower than that of the tea-garden coolies. The eosinophil percentage is above the figure given in British and American textbooks, but a little lower than we have previously found in Indian populations.

The mean of the platelet counts is exceptionally high, this seems to call for some comment. The blood was taken directly from the finger and placed on a slide on which a thin film of brilliant cresyl-blue had been deposited (*vide* Napier and Das Gupta, 1940) and covered with a coverslip. The reticulocyte count is done at the same time. By this method we have usually found higher platelet counts in normal individuals than the figures given in the textbooks.

ANALYSIS OF OTHER DATA

Bilirubinaemia — This was estimated by the indirect van den Bergh test: we used prepared cobalt sulphate solutions in sealed tubes for comparison. The results are shown in Table IV —

TABLE IV

Showing the result of the indirect van den Bergh reaction in different age groups

Age group Number of subjects			MILLIGRAMS OF BILIRUBIN PER 100 C.C. BLOOD		
			Less than 0.25	0.25 to 0.50	0.5 to 1.0
A	15	23	17	5	1
B	16	19	16	3	
C	14-19	64	50	13	1
D	20-24	31	23	7	1
E	25-38	31	25	6	
	14-38	126	98	26	2

There is no material difference between any of the age groups. In no case did we find a strongly positive van den Bergh reaction and only 2 showed over 0.5 mg (= one unit) of bilirubin. This is in sharp contrast to experience amongst tea-garden coolies where we found that 20 per cent of so-called normal individuals showed 0.5 mg or more, but it is in keeping with Mills and Mawson (1938) figures for normal individuals in Reading (England), the latter found only 4 out of 85 over 0.5 mg.

Fragility—The fragility test with different strengths of saline was carried out in 96 subjects. Table V shows the findings. There is a considerable degree of constancy in all the age groups, in the vast majority of subjects hæmolysis commenced at 0.38 per cent NaCl and was complete at 0.30 per cent, which is about the usual finding, though most textbooks give 0.42 for commencing hæmolysis.

TABLE V

Showing the percentage of saline in which hæmolysis occurred, i.e. the fragility test

Age group	Number of subjects	COMMENCING HÆMOLYSIS		COMPLETE HÆMOLYSIS	
		Mean	S. D.	Mean	S. D.
15	19	0.3811	0.0200	0.2916	0.0193
16	10	0.3920	0.0283	0.3020	0.0200
14-19	44	0.3859	0.0210	0.2955	0.0177
20-24	27	0.3748	0.0302	0.2985	0.0203
25-38	25	0.3712	0.0239	0.2928	0.0382

Erythrocyte-sedimentation rate (ESR)—This was done in 92 subjects, if 3 are excluded as being anæmic (*v s*), the remaining 89 showed a mean sedimentation of 29.52 ± 19.00 mm in one hour. This is a high sedimentation rate. The Graph shows the distribution of these findings. It will be seen that 10 subjects (or 11 per cent) had ESR above 50 mm, even after the anæmic subjects had been excluded. It is hard to believe that such a high percentage of apparently healthy individuals were suffering from concealed infections. Menstrual periods were excluded. The method adopted was the usual Westergren's technique. The blood was collected

separately in a flask in which the calculated amount of citrate had already been placed. If the data are corrected for cell volume by the Hynes and Whitby (1938) graph*, they can be grouped as shown in Table VI. In 30 per cent of the subjects the ESR was moderately or markedly increased. The youngest age group showed the lowest proportion of abnormal findings, but even in this group there were 27 per cent moderately or markedly increased, this is of course after correction has been made for cell volume.

GRAPH

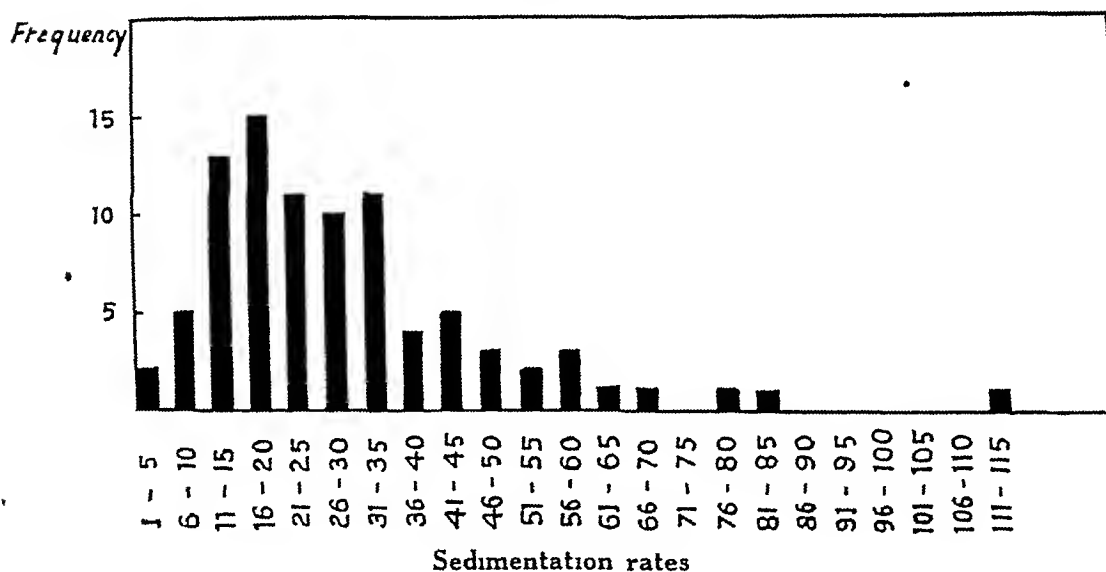


TABLE VI

Showing the distribution of the subjects according to their corrected erythrocyte-sedimentation rates (ESR)

Age group	Normal	Doubtful	Slight	Moderate	Marked	TOTAL
			Increase			
14-19	13	14	6	7	5	45
20-24	2	2	5	8	6	23
25-38	4	4	5	3	5	21
All ages	19	20	16	18	16	89

* This graph was not based on Westergren's technique, for which no correction formula appears to be available, but it can be adopted as a rough means of correction.

We correlated these findings with the degree of anæmia (Table VII), it will be seen that there is a clear correlation between low hæmoglobin and a high ESR. This would of course be much more marked before the correction for cell volume had been made.

TABLE VII
Showing the correlation between ESR and hæmoglobin percentage

ESR	All subjects	Above the mean hæmoglobin	Below the mean hæmoglobin
Normal	19	9	10
Doubtful	20	11	9
Slight increase	16	9	7
Moderate „	18	7	11
Marked „	16	4	12

This does not in our opinion indicate that all these women had some unrecognized infection though some of them probably had, but rather that the standards for the ESR require revision for application to Indian women. Sufficient emphasis has not been laid on the effect of temperature. Wintrobe and Landsberg (1935) have shown that temperature markedly affects ESR. In a table they show that as the temperature increases from 72°F to 90°F the ESR increases by 7 mm. Our laboratory temperature is often much above the latter figure and these examinations were all carried out in hot weather. This will therefore further help to explain the high readings*.

*The high erythrocyte sedimentation rates have been questioned by a colleague. The blood was collected in a flask and well mixed before being placed in the sedimentation tubes, but there was some delay, an hour or so, between the time that the blood was taken and the time it was put up into the sedimentation rack. We do not believe that this is a very important factor. However, since writing this paper we have tested the effect of temperature on the readings and find that in the 98°F incubator the sedimentation is often 10 mm to 15 mm greater than in the cool room, 75°F to 78°F. Our summer temperature, when these tests were made, is often above the former figure and our winter temperature below the latter. We also tested the effect of delay in carrying out the sedimentation test at a high temperature. With one half of the sample we did the sedimentation test at 98°F, the other half was kept in the same incubator for 2 hours, thoroughly re-shaken, and then placed in the sedimentation tubes and kept at 98°F for an hour. In 12 cases, in which the sedimentation rate varied between 7 mm and 119 mm, the mean of the first series was 46 and that of the second 53.

Allowance should be made for both the temperature and the delay factors, if the results are to be comparable with other normal findings, but it must be remembered that these are the circumstances under which the test is frequently made in India. Even if a full allowance is made for these factors the range of the sedimentation rates we are reporting is much higher than that given in the textbooks.

Our results are given for what they are worth. At least they convey one lesson, namely, that a high ESR in a woman in Calcutta should not *per se* be taken too seriously.

Wassermann reaction—This test was done on 124 subjects. The incidence of positive findings was about that usually found in the general population in India, namely 12.1 per cent. Table VIII shows the findings in different age groups. The difference between groups is not 'significant'.

TABLE VIII

Showing the Wassermann reaction in subjects in different age groups

Age group	Negative	Doubtful	Positive	TOTAL
14-19	42	14	7	63
20-24	23	4	5	32
25-38	21	5	3	29
All ages	86	23	15	124

Social, religious and racial differences married or single—Only 12 women were married and of these 7 were in the oldest age group, the means of their hæmoglobin percentages was 87.71. This is less than the mean of the age group, but the difference is not significant as the number is so small.

Religious groups—Only Hindus and Indian Christians have any considerable representation. The hæmoglobin data are shown in Table IX, though in all three groups the Christians have the highest hæmoglobin, the oldest age group shows the most marked difference but this difference is not significant.

TABLE IX

Showing the mean hæmoglobin in different age groups according to religion

Age group	Religion	Number of subjects	Mean hæmoglobin
14-19	Hindu	31	89.68
	Christian	31	90.10
20-24	Hindu	15	89.53
	Christian	17	91.82
25-38	Hindu	14	87.50 \pm 11.82
	Christian	12	92.00 \pm 7.11

Province of origin—Very naturally the majority of the subjects came from Bengal, in fact, in the younger age groups almost all came from Bengal. On the other hand, of the 31 subjects in the oldest age group only 11 came from Bengal. The other provinces represented were the Punjab, 6, Central India, 4, the United Provinces, 4, Assam, 3, and Bombay, 3. The mean hæmoglobin percentages for each of these groups has been worked out (Table X), but the numbers in most cases are too small to make comparison worthwhile. However, the mean for the Bengal women of this age group is strikingly low and is 'significantly' lower than the

TABLE X

Showing the mean hæmoglobin in different age groups according to whether they originated in Bengal or not

Age group	Province	Number of subjects	Mean
14-19	Bengal	59	89.73
20-24	Bengal	28	90.75
	Other provinces	6	92.17
25-38	Bengal	11	84.73 \pm 11.09
	Other provinces	20	93.60 \pm 7.36

mean of the subjects of all the other provinces grouped together ($\frac{m_1 - m_2}{S.E.} = 2.39$). This racial difference in the composition of the age groups makes comparison between them of little value. As far as hæmoglobin was concerned, it will be remembered that there was no significant difference between any two groups. Even if Bengal subjects only are taken the difference is still not 'significant'. This observation emphasizes two facts: one that from the age of about 15 women show no appreciable increase in hæmoglobin and, secondly, that women in Bengal show a lower hæmoglobin level, not only lower than women in Europe and America but also lower than women in other provinces in India. From the Bengal point of view, therefore, the mean hæmoglobin of all subjects in this investigation has been raised by the inclusion of women from other provinces.

Diet—The data collected regarding diet was too vague to be of any value.

SUMMARY

1. Hæmatological examinations were carried out in 134 apparently normal women in Calcutta. Hæmoglobin, total red cells, reticulocytes, corpuscular values, total white cells, eosinophils, the Arneth count, platelets, van den Bergh

reaction, fragility of the red cells, erythrocyte-sedimentation rate and Wassermann reaction were the examinations carried out

2 Six subjects were disqualified as being anæmic, in the remaining 128 subjects the mean and standard deviation of the findings is given in a tabular form

3 Analysis according to the age of the subjects suggests that there is little change in the blood findings in women after the age of 15, except that the leucocyte count shows a progressive decrease and the Arneth count weighted mean a progressive increase with age. The Bengalee women show a 'significantly' lower hæmoglobin than other Indian women included in this inquiry

4 The range of the erythrocyte-sedimentation rate is much higher than the figures given in the literature indicate is the normal for women

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The writers' thanks are due to all those who helped them, not only those whose names have been mentioned above, but to the subjects themselves and the superintendents and matrons of the institutions from which they came, for their willing co-operation

Our thanks are also due to Lieut-Colonel S D S Greval, I M S, Imperial Serologist, Calcutta, for doing the Wassermann reaction in 124 of these subjects

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THE BEHAVIOUR OF THE INFECTIVE LARVÆ
OF *WUCHERERIA BANCROFTI* WITH SPECIAL
REFERENCE TO THEIR MODE OF
ESCAPE AND PENETRATION
OF SKIN

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THE knowledge available on human infective larvæ is of very recent origin. Abe (1937) reported on the general behaviour of the mature larvæ and Menon and Iyer (1936) reported on the viability of the larvæ and the possibility of water infection. Abe (1938) has shown that larval penetration of intact skin is not possible under experimental conditions and Yokagawa (1939) studying the mode of infection thought that successful transmission of the larvæ by the mosquito was a matter of chance and that water was unlikely to play any part in the transmission of the disease. The mechanisms of larval escape from the labium, successful larval entry into the tissues of the host as well as the possibility of indirect transmission through water were therefore studied as further stages in the problem of transmission.

MATERIAL

Infective larvæ were obtained by artificially infecting laboratory-bred mosquitoes. The mosquitoes were released after a little starvation at night inside the mosquito nets of patients who were proved microfilarial carriers. They were collected in the morning after feeding. The mosquitoes were lethargic after a feed and so collection was easy. They were kept in mosquito 'hotels' throughout the

period of full larval development of 12 to 20 days, the period varying with season. The method of introducing the patient's hand into a small net was not successful, for, unlike the anopheles and the ædes which are much bolder in their feeding habits, *Culex fatigans* is very timid and is scared away by the slightest movement, never biting at all in the day. Methods in which the mosquitoes were enclosed in a beaker with the mouth closed with a net and applied directly to the skin of the patient as practised in malarial research work were not encouraging for obtaining feeds. Patients with a very heavy microfilarial rate were found unsuitable since a good percentage of the fed mosquitoes died in the first few days due to the severity of infection.

EXPERIMENTS AND RESULTS

1 *Movements of the larvæ in the mosquito* —The movements of the fully mature larvæ were studied by placing the mosquito, stunned by concussion and divested of wings and legs, on a glass-slide. Observation of the abdomen and proboscis was carried out through powerful transmitted light. In most cases larval movements could be well defined in the insect by this method. In cases where the labium was not transparent the inside stylets were very carefully separated from the labium for clearer observation. The larvæ exhibited worm-like translatory movements whether they were in the abdomen or in the proboscis. When the proboscis was brought in contact either with a cold or hot fluid the larvæ migrated back into the head and neck. Even normal saline at room temperature when in contact with the proboscis for any length of time made the larvæ migrate inside. This migration consisted in a simple backward movement of the larvæ without change of direction of the head-end inside the labium. Larvæ separated in saline showed only wriggling movements without active change in position.

2 *The possibility of larval escape into water* —This was next explored with reference to living and dead mosquitoes at laboratory temperature. The following methods were employed —

- (a) Water in a watch-glass was placed in each of the cages containing a batch of infected mosquitoes with fully developed larvæ. All other sources of food and water were previously removed. The water in the watch-glasses was examined for free larvæ at intervals. The results were all negative.
- (b) Mosquitoes showing 'proboscis' forms of the fully developed larvæ were stunned carefully and were placed in water in watch-glasses to observe if any larvæ escaped from dead mosquitoes fallen in water. Provided the body was intact no larvæ could be seen escaping out. All the larvæ died after a short interval of the death of the mosquito. But even a minute breach in the body of the mosquito, as would result from a leg pulled out, afforded a way out for the larvæ.
- (c) Water into which mosquitoes containing infective forms fell and died was examined for free larvæ. The results of repeated observations

were negative and always dead larvæ could be recovered from various parts of the dead insects and no free larvæ were ever seen outside

These experiments showed strikingly the difficulty of larval escape from the intermediate host, either during drinking or after death of the host

3 *The stimulus for larval migration out of the proboscis*—Cold, warm and hot saline, water, irritant fluids, fluid with the composition of human sweat, freshly drawn blood, mechanical bending of the labial sheath on the stylets as in the natural biting process were all studied with reference to living and stunned as well as recently dead mosquitoes. The method followed was to kill a mosquito with visible larvæ in its proboscis and to place it on a glass-slide under the microscope watching the effect of the various stimuli placed in contact with the intact proboscis. Warmth, irritants such as 1 in 1,000 dilution of alcohol and acetic acid, and the mechanical effect of bending the labium with a needle, all these caused visible increase in the larval activity inside the labium. If the fluid was too warm the larvæ either died *in situ* or migrated back into head and body regions. With moderate warmth (35°C to 37°C) the larvæ became very active and it could be seen that they repeatedly moved to the very tip of the proboscis in an attempt to rupture the membrane and escape out into the warm fluid outside. But no actual escape could be demonstrated. These experiments suggested that warmth was a definite factor in the larval escape. It caused increased activity.

TABLE I

Showing the effect of stimuli on the infective larvæ in the labium of the recently killed mosquitoes

Number	Stimulus	Effect
1	Normal saline at 29°C	After a few minutes of contact larvæ migrated back into body
2	" " at 36°C	Larvæ activated, the wavy curves change more frequently, repeated attacks on tip, but no escape
3	" " at 40°C	Larvæ intensely active at first, some migrated back into body, others soon lost activity
4	Water (tap) at 29°C, 36°C and 40°C	Behaviour same as with saline
5	Alcohol (1 in 1,000)	More active for a time
6	Acetic acid (1 in 1,000)	" " " " "
7	Blood (human), freshly drawn	More active at first, later sluggish
8	Fluid with composition of sweat	Behaviour as with saline
9	Mechanical pressure	Activity increased

The effect of warm blood and saline was especially studied when placed in contact with the proboscis of a *living* and stunned infected mosquito. In some cases the labium was pushed up on the stylets and kept bent a little in its proximal half with vaseline to imitate the natural position during biting when the stylets go down the skin. This experiment was repeatedly carried out with saline at 37°C, fresh human blood and rabbit's blood. Positive results were obtained on a few occasions. On all these occasions the mosquito was actively sucking up warm saline or blood placed on its proboscis with a bent labium. By the time a little distension could be seen in its abdomen due to the feed, it was observed that the larvæ escaped out into the fluid around the proboscis. Without this active sucking of warm fluid on the part of the mosquito the larval escape did not occur. On all these occasions, great care was taken not to injure the labium since, otherwise, the larvæ escaped out easily even into water at room temperature. From these experiments, bending of the labium and flow of warm fluid through stylets into the mosquito appeared to be the necessary stimuli for larval escape. These conditions are therefore an imitation of the natural processes. It was noteworthy that the larvæ appeared to escape on all these occasions through the extreme tip of the labella (Plate XI, fig 3).

4 *Behaviour of the infective larvæ outside the mosquito*—The larvæ, once they escaped into a fluid medium, lost their translatory movement and exhibited wriggling, coiling and uncoiling movements. They were heavier than water or normal saline and so sank soon after escape to the bottom of the container. In media such as blood partly clotted they regained their original migratory worm-like movements and travelled in all directions.

The larvæ did not show any special taxis effect to light or darkness, touch, pressure, slight variations of temperature or to the presence of lymph in the neighbourhood.

5 *Size, morphology and staining*—The larvæ when fully mature were of two varieties, the one slender and long, very active and well developed, and the other thicker, shorter, less active and herniating in water (Plate XI, fig 1). The latter probably represented maldevelopmental forms. The former were the typical infective larvæ and measured about 1,800/22 μ in the fresh state. The active and blunt end was made out to be the anterior one, while the posterior end was less active and distinguished by the presence of three well-formed papillary masses at the extreme tip (Plate XI, fig 2). The larvæ possessed a simple thin-walled alimentary canal running through the whole length of the body. The body-wall was relatively thick and the cuticle well developed and transversely striated. The protoplasm was semi-transparent in the living state. They could be stained by hæmatoxylin as well as carmalum when fixed, but all that could be made out was a granular structure of the thick body-wall, the inner structure remaining obscure. On the whole, staining was indefinite. In the living specimen the intestinal canal was thicker in its anterior third, abruptly narrowing at the middle third to a thin tube extending along the whole length. When alive the whole gut could be made out moving from side to side inside the transparent body-wall. Sex was indistinguishable.

PLATE XI

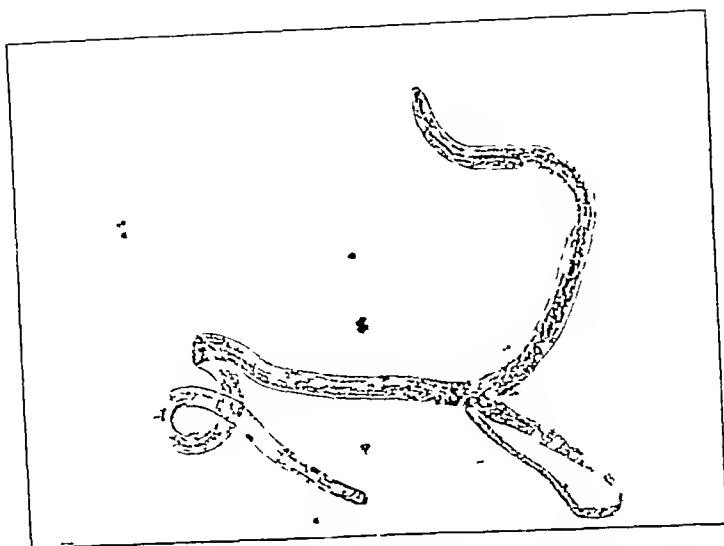


Fig 1 Intestinal herniation in a maldeveloped larva (15th day development) $\times 120$



Fig. 2 Posterior end of larva showing the papillæ $\times 260$



Fig 3 Escape of a mature larva from the proboscis under artificial stimulus of warm saline $\times 120$

6 *Viability and growth in vitro* —The viability of the larvæ depended mainly on whether the medium was sealed or kept exposed to the air. The nature of the medium used for preservation and the temperature influenced this to a lesser extent. Whatever the medium was, whether nutritive or otherwise, the period of viability was short in open surroundings such as in a watch-glass. But when the larvæ were enclosed in a chamber ringed round with vaseline and kept aseptically, viability was prolonged. Sepsis as in the case of microfilaria was fatal. Under aseptic conditions the larvæ could be kept alive for more than a week in human blood if the blood was daily renewed. Lower temperature certainly prolonged viability chiefly by retarding bacterial growth. The average viability in open surroundings was from 6 to 24 hours.

Though the larvæ survived for about a week in blood as well as in yolk of egg and were fairly active in both the media, no definite growth could be observed. Thyroid gland extract in great dilutions was employed with some media to stimulate growth. But the result was indefinite. It was common to observe granular degeneration in many specimens after a few days.

TABLE II

Showing the behaviour of the larvæ in various media in sealed chambers

Number	Medium	Temperature in degrees, C	Survival period.	Growth
1	Saline 85 per cent	29	12-24 hours	Nil
2	Blood (human)	29	4-8 days	
3	Blood (rabbit's)	29	2-3 "	
4	Yolk of egg	29	4-8 "	
5	White of egg	29	24-48 hours	
6	Hartley's broth	29	24-48 "	
	" "	37	24 "	
7	Hydrocele fluid	29	24-48 "	
8	Ringer Locke fluid	29	30 "	
9	Glucose broth	29	24 "	
10	Cooked meat medium	29	24-48 "	
11	Sterile milk	10	24 "	
12	Euphorbia milk	29	24 "	
13	Mosquito muscle tissue	29	24 "	

7 *Larval penetration of the skin*—Fully mature larvæ were isolated in minute drops of saline and dropped on to the skin by means of capillary pipettes. In the first series of experiments intact skin was used. The behaviour of the larvæ on the skin was viewed continuously through a binocular microscope until they died and notes were taken at definite intervals. The skins of rabbit, guinea-pig, mouse and man were all used for the experiments, which were repeated a number of times. The results showed that the larvæ were unable to penetrate intact skin. Even when the moisture around the worms was drying they failed to make any attempt to penetrate.

TABLE III

Showing results of experiments with intact skin

Number	Animal	Site	Duration of experiment, minutes	RESULTS
1	Rabbit	Skin of ear	5-20	Failed to penetrate and all were recovered dead
2	Guinea-pig	Skin of thigh	5-20	
3	Mouse	Skin of abdomen	5-20	
4	Man	Skin of dorsum of fingers and forearm	5-20	

It was decided therefore to study larval penetration on a broken surface of skin. In the initial experiments fine punctures were made on the skin with a sharp needle and the active larvæ were directly deposited on the punctured spot. All the animals in the series were employed one after another and the effect watched by direct observation through the microscope. The larvæ failed to make their way into tissues even through the artificial openings though serum was oozing out. During their movements they came directly over the punctures, but did not show any attempt to enter the tissues by their own movements. It appeared that serum had no special taxis on the larvæ, though like fresh blood it made the larvæ more active. The experiments were repeated with small cuts in the skin made with a sharp blade and larval movements were studied under the microscope. The results were again negative. However, when the larvæ were either dropped directly into the breach or were manipulated with a needle into the moist broken tissues they continued to live and be active. It was possible to observe their movements in the depth of the cut by traction on the two edges of the wound. Only when such exact localization was possible and there was no chance of drying, they migrated actively into the tissues of the breach and were lost to sight after varying intervals. It was also noted that they exhibited regular crawling movements on the moist tissues. No attempt could be observed suggesting a tendency to enter blood vessels but it was not possible to say whether lymph spaces were selected.

The results of these experiments suggested that infective larvæ did not possess the power of active penetration of intact skin or migration through broken skin, but required to be deposited or 'inoculated' into the tissues under experimental conditions. The fact that many larvæ could be recovered dead in the washings of the skin around an infected mosquito bite is in conformity with the results of these experiments. Both seem to show that larval deposition by the mosquito on the skin is more of an accident and that for infection an actual 'inoculation' of the larvæ through the labial sheath appears to be necessary. The nearest approach to the natural process could be obtained when the labium with infective larvæ was ruptured with a needle in the breach itself. Larvæ could be seen to emerge briskly and many disappeared into the cut while others strayed out and died on the relatively dry edges.

TABLE IV

Showing the conditions for 'taking' the infection

Number	Nature of breach.	Procedure	RESULT
1	Punctures with a needle	Five larvæ in saline dropped over	All larvæ died with drying, all recovered dead in washings
2	Small cuts	Five larvæ deposited over the spot	With drying of the part all the larvæ recovered dead in washings
3	" "	Ten larvæ 'inoculated' or manipulated into cuts	Larvæ alive, after an hour migrated out of view

DISCUSSION

A study of the results of these experiments suggests that the mechanism of transmission appears to be a complex process. It can be said to consist of two components, the first being the provision of a stimulus for the larvæ to penetrate the proboscis and the second a direct 'inoculation' of free larvæ into the puncture of the bite. The stimulus for the larval escape might be said to be *active sucking* of warm fluid or blood by the insect. This escape is also greatly aided by pressure changes in the labium caused by the bending of the part and the general rise of intra-coelomic pressure due to the feed itself. These could be studied separately and seen to cause increased larval activity as individual factors. Experimentally larval escape appeared to take place through the extreme pointed end of the labella. During a blood feed once the tip of the proboscis was punctured, the larvæ would find themselves directly in the puncture around the stylets in a small pool of warm

blood It is possible to visualize a larval descent right into the puncture depth alongside the withdrawing stylets But it is also equally certain that some larvæ stray out and find themselves on the relatively dry margins of the puncture These seem destined to die with drying of the part as they can be recovered in washings of these bitten areas Therefore under natural conditions insect transmission of filariasis appears not to result in a 100 per cent 'take' In the transmission of malaria with a direct injection through the stylets the chances of a positive 'take' are certainly much greater than in the haphazard larval escape into the puncture from a filariated mosquito Yokagawa (*loc cit*) also has suggested that filarial infection is a matter of chance It is interesting to note that in the literature on filariasis it is generally taken for granted that the infective larvæ, deposited on the skin by the mosquito, actively penetrate and enter the skin rather than seek for and enter the puncture left by the bite This was suggested by Fulleborn (1909) based on an analogy from the transmission of canine filariasis Recently, Abe (1938) has disputed this view with regard to the intact skin The experimental evidence in the present study shows further that the larvæ are incapable of penetration even through an abraded skin from their own migratory movements They require to be actually deposited in the moist tissues for survival and migration

To account for certain anomalies in distribution and symptomatology of the disease the probability of other modes of infection were put forward Water transmission has been put forward as an additional operative factor Our findings did not support these views The larvæ were not observed to escape into water either when the mosquito drank or died in water at laboratory temperature Larvæ separated artificially into water sank to the bottom and died in a short time or were an easy prey to mosquito larvæ and cyclops which destroyed them Under experimental conditions viability outside the mosquito was only for a very short time (6 to 24 hours) in open surroundings Further, the larvæ were incapable of affecting an entry through abrasions in the skin unless they are carefully deposited in the breach or 'inoculated' Obviously these are not conditions that could obtain in nature for the transmission of a disease, a matter of chance even after the bite of the insect vector

As the experimental work relates solely to *W bancrofti* the possibility of water transmission with other species has yet to be studied But with regard to bancroftian filariasis the present study tends to implicate the mosquito as the sole vector and the mosquito bite the only mode of infection

SUMMARY

1 Warm fluid at about 36°C around the proboscis causes increased activity of the larvæ in the proboscis and active attempts at escape

2 Actual larval escape from the proboscis occurs only under conditions similar to those obtained during the act of biting, i.e. the labium, pushed up on the stylets, is bent in its proximal half and warm fluid at about 36°C is sucked up through the stylets

3 Viability of infective larvæ is best in human blood and yolk of egg when kept in sealed chambers without exposure to air and free from sepsis. The maximum survival period obtained experimentally was more than a week, but no visible growth took place during this period.

4 Active infective larvæ under experimental conditions do not penetrate unbroken skin, but migrate through the tissues only when deposited on the moist tissues in a breach in the skin and do not appear to have any taxis when deposited in the neighbourhood or over a breach of skin surface.

5 The experiments do not lend any support to the view that *W bancrofti* can get into the human tissues from water through abrasions in the skin.

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DISTRIBUTION OF *V CHOLERÆ* AND EL TOR TYPE STRAINS IN CERTAIN RURAL AREAS IN INDIA

BY

W D B READ,

AND

S R PANDIT

(*Bengal Cholera Field Inquiry under the Indian Research Fund Association*)

(*All-India Institute of Hygiene & Public Health, Calcutta*)

[Received for publication, February 22, 1941]

THIS communication presents the results of an investigation into the distribution of the agglutinable* vibrio throughout the course of one year, 1939-40, in the general population and water sources of a rural area in one of the 'endemic' cholera districts of the delta of Bengal. The results of investigations into the water sources of certain other areas in India are controls for some of the findings in the water sources of the Bengal area and are also included.

Samples were collected by trained Bengali medical men with considerable bacteriological experience.

Results have had to be assessed in spite of many gaps and omissions. Each sample was submitted to a standardized technique and the results may be taken to indicate the facts as brought out by the method used.

AREAS EXAMINED

(1) *Bengal*—Two police station areas, 100 miles south-east of Calcutta, in the district of Khulna, 40 and 55 miles from the sea coast, were selected. The country under survey was 295 square miles and the population almost 200,000. The river system was tidal and the whole country was intersected by innumerable

* The term 'agglutinable vibrio' is used throughout to designate a strain agglutinable with pure 'O' serum of O group I (Gardner and Venkatraman, 1935) including sub-types Inaba and Ogawa.

water channels, communication being largely by field paths and boat. In the northern half of the area there were earth roads and a light railway, while the number of water channels was also reduced. The water-supplies, except in the one municipal town of the area, which was excluded from the investigation, were from rivers and tanks, which especially in the southern part of the area were usually in communication by ditches and khals with the main river and were subjected to an automatic flushing action with the rise and fall of the tide. In the rainy season the country was liable to inundation and the flow of water in the main river was towards the sea at all states of the tide.

The population consisted chiefly of field labourers or fishermen, while chiefly Mohammedan, Hindu villages also occurred. It was largely non-migratory.

The areas were selected on the basis of the following points —

- (a) High general incidence of cholera in the previous 5 years
- (b) A constant seasonal variation in the occurrence of cholera in the area, i.e. cessation or reduction in the rainy season. (This is typical of a large part of Bengal.)
- (c) Local reports as to the 'salinity' of the water
- (d) Non-migratory habits of the population
- (e) Available water sources not under supervision

(2) *Bihar* — This station was selected as representative of an epidemic area, in which severe cholera is an annual occurrence and in which the period of maximum incidence as opposed to that of Bengal is during the monsoon season. The area was heavily watered by the Ganges and its tributaries and being situated high up from the mouth of the river was not affected by tidal influences. Moreover, the usual water-supply was from shallow wells.

(3) *Sind* — This station was selected in a part of a province which for the last 10 years has been very largely free of cholera. In the area selected there had been no cases for over 10 years. Water-supplies were from tanks, wells, lakes, marshes, streams and rivers. The tanks were filled yearly by the rise of the Indus in flood and were then shut off from the river connections for the rest of the year. The winter climate was mild and the station being adjacent to the Indus delta some of the water-supplies were brackish. The water-flow in the jhils and streams was very sluggish in the non-rainy season.

TECHNIQUE

Stools — These were passed in the early morning by selected individuals into marked earthenware pots with lids, and were collected often within a few minutes and always within 2 hours. A sample was removed with a bamboo stick into 50 c.c. of 2 per cent common sea-salt solution in a wide-mouthed glass-stoppered bottle, so as to obtain a thick emulsion. In many of the samples collected, 3 to 4 drops of N/1 NaOH were added to the saline before collection, owing to the acidity found in the average stool. Media bottles were inoculated with the stool suspension

in the local laboratory usually within 2 hours. The technique recommended by Read (1939) was followed except that the liq. bismuthi was reduced to 0.04 c.c. The bottles were then despatched to the headquarters laboratory at Khulna and were ready for plating 24 hours later. The mannose solution used was manufactured in the laboratory from ivory-nut-shavings obtained from America, according to the method of Bose (1939) and Narayanan (1941). Allowances in the media formula had to be made owing to the reduced concentration of mannose obtained in the rough extracts (4 per cent to 6 per cent). The ingredients used for test were checked by weekly isolations from artificially inoculated stools.

pH of stools—This was estimated by a rough method similar to that recommended by Read (*loc. cit.*) for adjusting the pH of media to 9.2, bromo-cresol-purple in 96 per cent alcohol (0.04 per cent) and 0.02 per cent phenol red being substituted for thymol blue. 1.5 c.c. of a stool emulsion whose opacity did not appreciably interfere with the colour reaction was used in small test-tubes and to this 6 drops of indicator were added. A little experience enabled correct recognition of the various colours indicative of different pHs, but the results obtained were of course approximate.

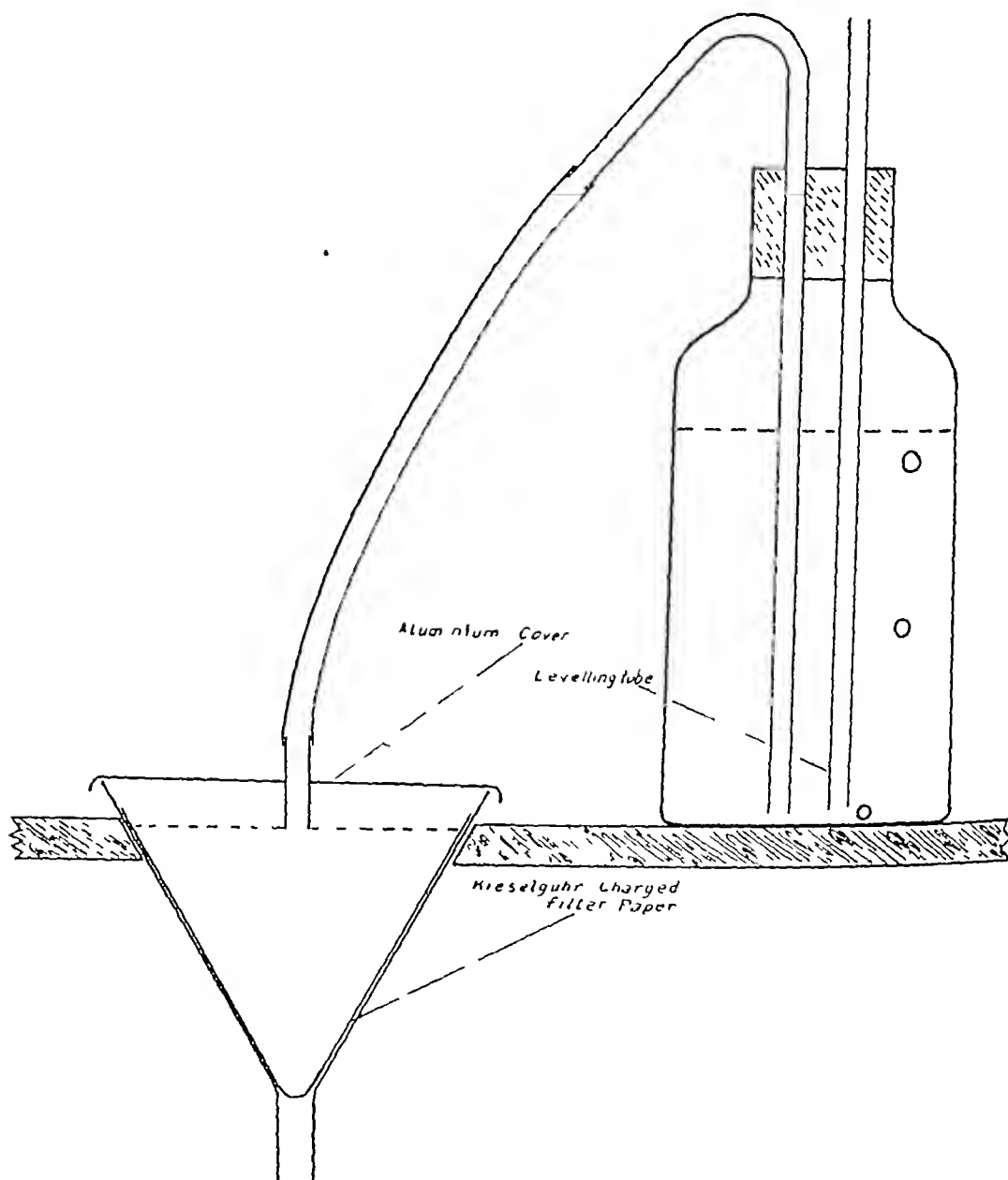
Waters—1,500 c.c. of water from each source were collected in sterilized whisky bottles. To 1,000 c.c. was added by means of a scoop, common bazaar sea-salt so that the salt concentration was about 1 per cent and sufficient alkali to raise the pH to 9.2. This was then filtered through Kieselguhr impregnated filter-papers by the method recommended by Read (*loc. cit.*), the water being poured through funnels by hand. In the Bihar and Sind investigations an improved method consisting of the use of funnel covers and automatic levelling apparatus similar to that described for phage filtration by Pandit (1934) was used (*vide* Sketch). After filtration, the filter-papers were folded in small packets with due regard to asepsis and covered consecutively with (a) cellophane, (b) vaseline paper, (c) cellophane and (d) ordinary brown paper. The cellophane and paper used were sterilized by autoclaving. The paper was dipped into sterilized vaseline, allowed to cool and excess of vaseline removed. The packet was then ready for despatch to the central laboratory for inoculation into the bismuth sulphite medium. The ingredients used for test were checked by weekly isolations from artificially inoculated water.

Blood—Sera for agglutination were separated in the field and sent into the main station in glass ampoules. Tests were put up in round-bottomed tubes against formolized and boiled suspensions of Inaba and Ogawa type vibrios (TMCH 1,800/1 and PIS 653). Readings were taken after the tubes had remained 2 hours in the incubator and a further 22 hours at room temperature. Trace readings were neglected.

Hæmolysis—Strains were tested by the method recommended by Greig (1914) except that the Douglas broth (Douglas, 1914) with 0.85 per cent NaCl was utilized for growing the organisms.*

* In the case of certain of these strains, isolated in the first half of the year, the test by the above technique was not carried out till 6 months or less from their date of isolation, but many repeat tests since that date have failed to alter the findings.

SKETCH SHOWING THE IMPROVED METHOD OF FILTRATION



ISOLATIONS OF NON-HÆMOLYTIC AGGLUTINABLE VIBRIOS.

Stools—Table I gives results of isolations from different sources

Of the four cases from the + areas, not proved positive, three were examined first on the 7th, 16th and 17th day after the onset of the disease, respectively One

case vibrio proved hæmolytic. This bore the same number as a non-hæmolytic water strain that was isolated in the same week from the same area. It seems that confusion was very likely but there is no direct evidence for this. Of the C_1 contact stools all except one that were found positive occurred in three areas where the percentage of vibrios found in the population examined was 7, 10 and 19 per cent. There was a total failure to isolate *V. cholera*.

TABLE I

Isolation of non-hæmolytic agglutinable vibrios from stool sources in Bengal stations

	Number of sources examined.	Inaba sub type	Ogawa sub type
NC	3,003	0	0
Case (+ areas)	32	0	*19
C_1 "	316	0	13
C_2 "	278	1	0
Case (- areas)	12	0	0
C_1 "	140	0	0
C_2 "	218	0	0

Abbreviations used—

NC—Specimens collected in the absence of adjacent clinical cholera

C_2 —Specimens collected from individuals or water sources in a village in which cases of cholera were occurring or had recently occurred

C_1 —Specimens collected from individuals and water sources of the same family or house with cases of cholera or otherwise closely connected.

Cases—Those exhibiting symptoms of cholera or diarrhoea

+ areas—Those areas in which the non hæmolytic vibrio was actually isolated

- areas—Those areas in which there was no such isolation.

* One of these vibrios was found hæmolytic but in this connection see text.

from the stools of individuals not in contact with cholera. In this latter connection see Table II giving the pH found in normal stools. As there was considerable variation found in the two field stations in the Bengal area, the results from these two stations are given separately. In one station about 20 per cent of stools had a pH of 6.0 or less and over 50 per cent had a pH of 6.6 or less. In the other station 50 per cent of the stools had a pH of 6.0 or less while 85 per cent had a pH of 6.6 or less. The significance of these findings is at present

under investigation It will be noted that the addition of 3 to 4 drops of alkali to the saline brought the pH of nearly all stools to the level of 6.8 or over

TABLE II

*Approximate pH of stools collected from the Bengal stations
in 2 per cent saline*

pH of stools —	5.2	5.4	5.6	5.8	6.0	6.2	6.4	6.6	6.8 and over
<i>North station —</i>									
Collected in neutral saline } Numbers found	5	12	15	36	58	46	60	70	294
Collected in neutral saline with 3 drops N/1 NaOH to 50 c c stool emulsion } "						3	14	7	779
<i>South station —</i>									
Collected in neutral saline } Numbers found		16	14	12	7	20	9	7	15
Collected in neutral saline with 3 to 4 drops N/1 NaOH to 50 c c of stool emulsion } "		5	3		1	1			184

Waters — Table III gives the incidence of non-hæmolytic vibrios found in waters —

TABLE III

*Incidence of non-hæmolytic vibrios found in waters in epidemic areas
in Bengal stations*

	Number of sources examined	NUMBER OF SOURCES POSITIVE	
		Inaba sub type	Ogawa sub type
C ₁ (+ areas)	85	7	7
C ₂ "	170	0	3
C ₁ (— areas)	31	0	0
C ₂ "	117	0	0

In addition to vibrios enumerated in Table III, non-hæmolytic vibrios were isolated from two sources not in contact with cholera. Of these, however, there is a possibility that one was confused with a hæmolytic case vibrio as noted above.

The non-hæmolytic vibrios were therefore with one exception confined to areas in immediate contact with cholera cases.

Water specimens numbering 3,042 from 1,442 sources were examined in Bengal in the course of the year.

From the Bihar station from cholera free villages in a cholera infected area during the epidemic season, 515 specimens from 397 sources proved negative for *V. cholera*.

From the Sind station 528 specimens from 205 sources from villages that had been free of cholera for over 10 years proved negative for *V. cholera*. Examinations were carried out in November and December 1940.

EVIDENCE FOR THE PERSISTENCE OF THE NON-HÆMOLYTIC VIBRIO

Ten non-fatal cases of clinical cholera were followed up. Of these three were found positive on the 8th, 9th and 13th day from the onset of the disease, while the remainder were not found positive after the 5th day. One case was proved vibrio-free in 3 days.

Sixteen contacts that were shown positive were followed up. Of these only four remained positive after 5 days from the date of onset of the last case of clinical cholera occurring in the family.

Seventeen positive water sources were also followed up. Of these nine were shown to be positive after 5 days from the onset of the last connected case found positive.

The longest period of survival found in cases was 13 days, in contacts 9 days and in waters 16 days. The above particulars are given with the proviso that a continuous series of isolations was not possible in the case of all the sources examined. At the same time in nearly every case a series of negative specimens taken at intervals confirmed the statements given. On the results obtained it seems that examination of individuals who have not proved positive during the first 5 days after the onset of the last connected case is unlikely to detect carriers. In no case was there any evidence of water infection preceding an outbreak though provision for the collection of adequate data on this point could not be made. The usefulness of the figures given for persistence in the stools of contacts and in water depend on the assumption that the most obvious source of infection was the actual and that the water sources were initially infected by cases and not vice versa. Both contact and water sources may, however, have been infected from sources outside the cases immediately connected with them.

Subsequent to the epidemic year in which the above investigation was carried out, 144 water samples were examined from villages in which cholera had been present during the previous year and none of the samples showed *V. cholera*.

Table IV gives the number of cholera cases reported from each village. Where epidemics of 11 and 12 cases are listed, these refer to a union of villages where the population would be four to five times that of other villages. The average village population may be taken as 300. It is worth while noting that in villages where there was bacteriological evidence of cholera except in two outbreaks, both consisting of a single imported moribund case, there was always evidence of at least one connected case. Cases were by no means confined to one compound or family though in any one outbreak a definite part of the village was usually alone infected, large areas in the same village remaining free. In villages in which cases were reported and there was no bacteriological evidence of cholera there was nothing definite to suggest a diagnosis of cholera on general grounds and the number of cases occurring never exceeded two.

TABLE IV

Number of cases of cholera reported in each village

Epidemics of	cases	1	2	3	5	6	7	11	12
Number of villages affected	+ areas	2	1	2	1	1	2	1	1
do	do	8	5						

Table V gives the duration of the outbreaks in each village. This duration could not be related to the geographical distribution of the population, to the number of cases, to the infection of water, or to any other factor. In villages where there was no bacteriological evidence of cholera, the duration of an outbreak exceeded one day in two instances only. In the case where the epidemic lasted 6 days, there were two diarrhoeal cases only and where it lasted 3 days, while one reported case was fatal the second one was a diarrhoeal case only. While in several instances the outbreak was assignable to traceable causes, in the majority no evidence was obtained of its origin. This point was investigated with care.

TABLE V.

Duration of outbreak in each village

Duration in days between onset of first and last cases	1	3	4	5	6	16	17	18
Number of villages affected	4	0	1	1	0	1	2	1
do	11	1	0	0	1	0	0	0

ISOLATIONS OF THE HÆMOLYTIC AGGLUTINABLE VIBRIO (EL TOR TYPE)

It was not expected that vibrios of this type would be found in the course of this investigation as they had never been reported from Indian sources previously. However the first vibrio was isolated within a fortnight of commencing work.

Bengal—Table VI gives the number of water sources examined month by month, the number which proved positive and the serological sub-types to which they belonged. The findings in the two field stations are given separately. Each source has been treated as a single source for a particular month but when examined in any following month it has been taken as a fresh source for that month.

TABLE VI

Isolation of hæmolytic agglutinable vibrios—monthly incidence

BENGAL

	NORTH STATION			SOUTH STATION		
	Number of water sources examined	SOURCES POSITIVE		Number of water sources examined	SOURCES POSITIVE	
		Inaba sub type	Ogawa sub-type		Inaba sub type	Ogawa sub type
June 1939 -	42	0	0	60	0	0
July 1939	48	0	1	103	2	10
August 1939	81	0	3	56	1	11
September 1939	72	0	2	140	0	5
October 1939	36	0	3	96	0	3
November 1939	102	4	0	128	0	3
December 1939	84	0	0	157	0	2
January 1940	120	0	1	154	0	1
February 1940	74	0	0	186	1	1
March 1940	94	0	0	74	0	0
April 1940	52	0	0	75	0	1
May 1940	38	0	0	53	0	0

It will be seen that the hæmolytic agglutinable vibrio in one station was isolated in the months July to November and in the other almost throughout the year. In both stations there was a higher percentage of isolations in the rainy season.

Taking *actual* sources, out of 763 such examined during the year in one station 13 were positive, i.e. 1.7 per cent, the corresponding figures for the other station were 29 positives out of 679, i.e. 4.3 per cent

All these vibrios with the exception of those indicated in Table VII were isolated from sources in villages from which cholera was absent. It seems likely that those isolated during epidemic periods were present in the water-supplies owing to the same causes responsible for their presence in uninfected areas

TABLE VII

Incidence of the hæmolytic agglutinable vibrio in the presence of the disease

Bengal	Number of sources examined	Number of sources positive
C ₁ + areas	85	2
C ₂ „	170	1
C ₁ - areas	31	3
C ₂ „	117	1

Other stations—In the Bihar station the monsoon was in progress during the investigation. Altogether from the 397 sources examined, four sources proved positive including one river

In the Sind station 206 sources were examined and 18 proved positive

EVIDENCE OF PERSISTENCE OF THE HÆMOLYTIC VIBRIO

In the Bengal station from one tank isolations of the same type of hæmolytic vibrios were made for a period of 19 days taking samples at intervals, 6 out of 10 samples collected during the period being positive. In another tank isolations were effected over a period of 35 days but for a period of 3 weeks in the middle of this period three specimens proved negative. It is to be remembered that tanks in this area were subject to an automatic flushing from the rise and fall of the tide

In Bihar on the other hand two wells remained positive for 22 and 30 days respectively, while a third well and the river specimen were rapidly cleared

In Sind one tank proved positive over a period of 41 days, eight out of eight specimens collected during the period being positive. Two jhils were positive over 35 and 41 days respectively, four out of eight specimens proving positive in the former and three out of four in the latter. In other positive sources repeated examinations showed no evidence of persistence

CHEMICAL ANALYSIS OF WATER

Nearly all water samples were chemically analysed to determine the amount and nature of their salt and organic matter content with special reference to the quantity of sodium chloride present and any possible effect of sea-water on the water-supplies in the area of investigation. This was done in all the three provinces. The results are not reported in this communication as it was not possible to establish any constant relationship between the bacteriological and epidemiological findings and the chemical nature of water-supplies in the areas in question.

AGGLUTINATION TESTS ON HUMAN SERA

The frequency distribution of positive results at different dilutions of sera obtained from unimmunized individuals in the three provinces is given in Table VIII.

It is clear that, with the technique adopted, no evidence of immunity has been obtained from a study of agglutinins in blood in the general population of Bengal and Bihar as compared with that of Sind. It may be stated here that almost all positive reactions were partial.

GENERAL FINDINGS

The distribution of the agglutinable vibrio, both hæmolytic and non-hæmolytic, on the basis of a fixed isolation technique has been worked out in an endemic rural area in Bengal and two areas in Bihar and in Sind which are as nearly similar as possible, geographically speaking and otherwise, to the area chosen in Bengal but differ in their epidemiological history. The non-hæmolytic agglutinable vibrio was found in all except one of the clinical cases in areas where the presence of cholera could be established, provided the examination was carried out sufficiently early in the disease. About 7 per cent of close contacts of cholera proved positive and about 16 per cent of water sources in direct contact with cases were positive at different periods of the epidemic. There was no evidence of persistence in any source much over one fortnight and the majority of the sources rapidly became free of the vibrio. On the other hand the non-hæmolytic vibrio with one or possibly two exceptions was not found in the absence of the disease.

The hæmolytic agglutinable vibrio, while detected in the presence of the disease, has been found usually in its absence. It has been found in cholera areas of two different epidemiological types in different provinces of India and in relatively large numbers in an area which must be taken as not only free from cholera during the period of investigation but free from cholera during the decade previous*.

The findings in these respects so far are out of line with those described in Celebes Islands (de Moor, 1939).

* Dr K V Venkatraman working in the district of Tanjore, Madras, has also isolated El Tor type strains from tanks in the absence of cholera (personal communication).

TABLE VIII.

Showing the frequency distribution at different stations of sera from unvaccinated individuals

Place	Number of sera examined	Serum dilution —		Inaba sub type				Ogawa sub type			
		Suspension employed —		- 1/25	+ 1/25	+ 1/50	+ 1/125	- 1/25	+ 1/25	+ 1/50	+ 1/125
Khulna	248	Formolized		220	19	6	3	218	16	13	1
	145	Boiled		113	18	11	3	98	27	17	3
Calcutta	39	Formolized		8	19	12	0	7	12	20	0
	39	Boiled		14	10	15	0	8	16	15	0
Patna	50	Formolized		23	15	9	3	20	11	19	0
	47	Boiled		22	15	9	1	18	16	9	4
Sind	50	Formolized		29	18	3	0	38	9	2	1
	17	Boiled		8	4	5	0	7	1	8	1

It is not possible at this stage to decide whether or not the findings with regard to the incidence of the non-hæmolytic agglutinable vibrio represent the actual degree of prevalence or whether the technical difficulties of isolation have proved too great to allow of the isolations of small numbers of the agglutinable vibrio from natural sources. Work is in progress to determine what are the limits down to which it is possible to isolate freshly isolated case vibrios from artificially inoculated specimens of normal stools and raw waters. At the present time it appears that there are limits both in stools and waters beyond which the reaction of the stool and the presence of other organisms in water will prevent isolations by the technique adopted.

The staff in this investigation consisted of —

S R Pandit, W D B Read, D N Chatterji, N M Moitra, S C Seal (later H S Sinha), P C Das, S Bose (later E K Narayanan and N K De) and B C Chakrabarty

ACKNOWLEDGMENTS

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APPENDIX

A village outbreak of cholera in Bengal

In order to appreciate more clearly what is happening in a village epidemic of the type that has been under investigation in the work of the Bengal Cholera Field Inquiry, a description of one outbreak is detailed here. The population of the total area examined was about 2,000 spread over a distance of $3\frac{1}{2}$ miles, there being houses at close intervals throughout this area.

Eleven cases in all occurred in the space of 13 days of these seven were fatal. Six were bacteriologically examined and six were found positive for the *Inaba non-hæmolytic vibrio*. One hæmolytic vibrio was also isolated from water but all isolations from other sources were of the *Inaba non-hæmolytic* type. Four of the vibrios were isolated from family HM, one from family FH and one from family JAF (*vide* Diagram). The first case, a child, occurred in the family of KH on 22nd February, 1940, and died on 24th February, 1940, the second also a child in a family resident 250 yards from the former contracted the disease on 25th February and died on 26th February. The third and fourth cases occurred on 29th February in the family to which the first child belonged of these one proved fatal and one recovered. The first investigation was made on 29th February, 1940 seven days after the occurrence of the first case. Eight contacts from the family of KH and three water samples proved negative, but from the family of HRS one out of thirteen contact stool samples was positive and four water samples were negative, also a tank used by a family resident 200 yards from HRS was positive for the hæmolytic vibrio. The position on 29th February was thus —

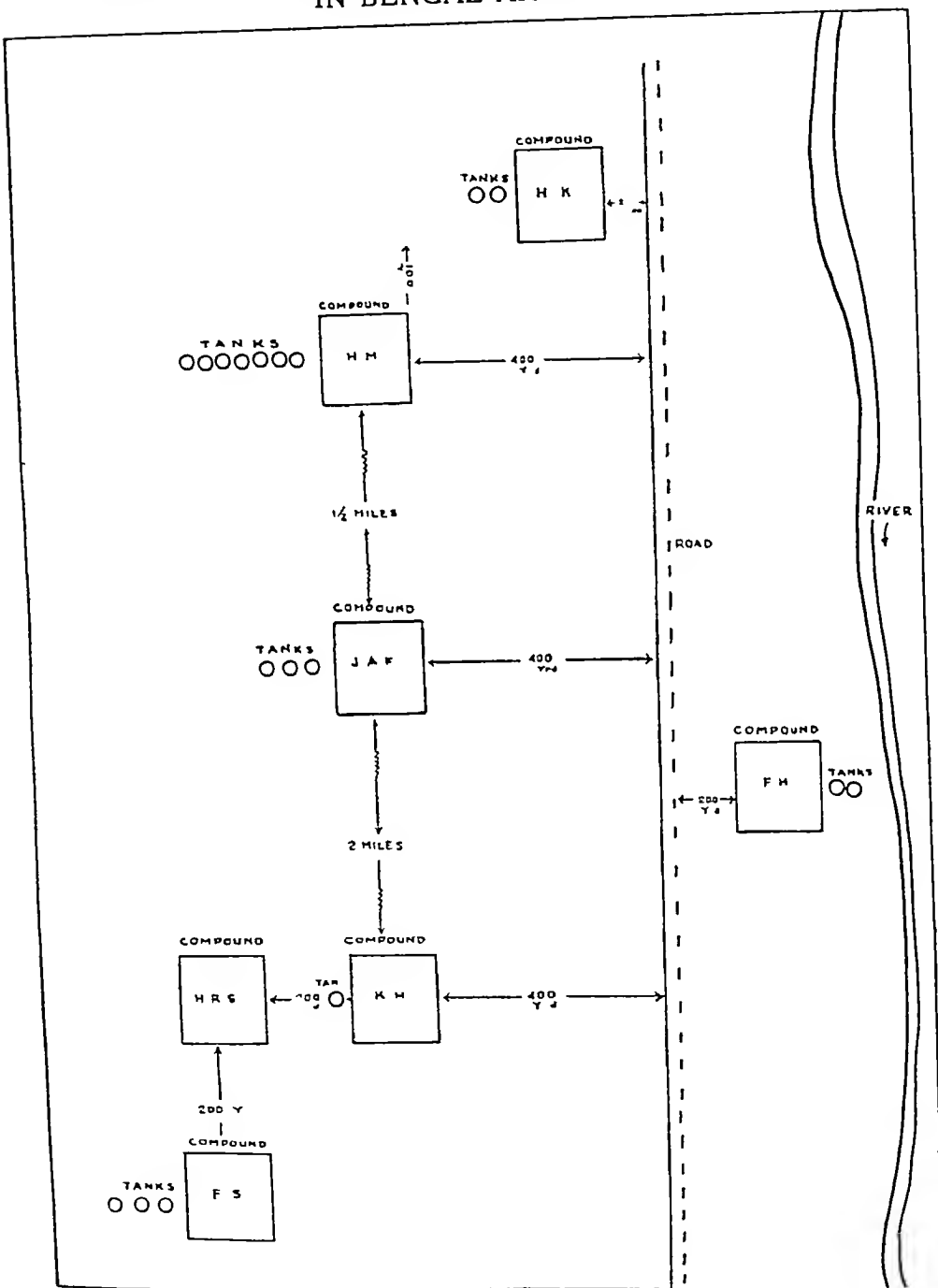
Family	CASES		CONTACTS		WATERS	
	Number occurring	Positive	Number examined	Positive	Number examined	Positive
KH	3	(Not examined)	8	0	3	0
HRS	1	„	14	1	4	0
FS	0	0	0	0	1	1 (hæmolytic)

The bacteriological proof of cholera at this stage had to be established from one contact case only, no cases being available then or later for examination. On 6th March, 1940, one further contact was found positive in the family of KH.

Two collectors and four sweepers were employed and there was no question of slackness on their part. The facts are illustrative of the difficulties of establishing a diagnosis under the conditions of work in India.

W D B Read and S R Pandit
 DIAGRAM ILLUSTRATIVE OF VILLAGE OUTBREAK
 IN BENGAL AREA

417



On 4th March, 1940, at a distance of $3\frac{1}{2}$ miles away, a case occurred in the family of HM. This proved fatal the following day. A further case occurred in the immediate neighbourhood on 5th March, 1940, which proved fatal four days later and three more on 6th March, 1940, of which two recovered. Only two of these five cases occurred in the same house, the remainder being one per house. On 5th March, 1940, also, a further fatal case occurred in a house $1\frac{1}{2}$ miles distant. This individual had had contact with the family of HM. There was, however, no traceable contact between the family of KH and HM.

Investigation in the area of family HM was started one or two days before the onset of the last four cases. Only one case had occurred previously. Twenty-two stools of individuals without symptoms were examined and of these three were found positive. Of the latter, one, two days later, developed symptoms and died, the two other remaining without symptoms throughout the epidemic, though one of these was again positive two days later. One water sample out of eight examined proved positive.

Further investigations were carried out on 6th March, 1940. From the family of HM, four cases proved positive and three out of eleven contacts were also positive. From the family of JAF, the case proved positive, while three contacts were negative. On 7th March, 1940, one water sample from the latter family was positive. On 6th March, 1940, one contact was found positive in a family resident at 100 yards from HM.

The position on 7th March may be summarized as follows —

Family	CASES		CONTACTS		WATERS	
	Number occurring	Positive	Number examined	Positive	Number examined	Positive
HM	5 (4 examined)	4	22	4	7	1
JAF	1	1	3	0	4	1
HK	0	0	9	1	3	0

After this no further cases occurred. One case however, in the family of FH, continued to excrete the vibrio till 18th March, but was shown to be negative on 28th March and thereafter. Further positives were recorded as follows —

Family	JAF	Contact	1	Positive	11-3-40
„	HM	Water	1	„	10-3-40
		Water	1	„	13-3-40
		(another sample)			

The total number of specimens examined was—

Cases	20
Contacts	269
Waters	104

also many specimens from sources not in direct contact with the disease

OCCURRENCE OF VIBRIO EL TOR IN NATURAL SOURCES OF WATER IN THE ABSENCE OF CHOLERA

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THE report of an outbreak of cholera in the Celebes attributed to vibrio El Tor (de Moor, 1938) has created a renewed interest in the question of the significance of this vibrio in the epidemiology of cholera. De Moor (*loc cit*) isolated hæmolytic agglutinable vibrios* from 36 cases presenting clinical symptoms of cholera, 29 healthy contacts, and 17 sources of water in immediate relation to the positive cases. He also obtained them from 4 persons (non-contacts) resident in the affected villages. This vibrio could not be recovered in numerous examinations of stool specimens from parts of the islands not involved in the outbreak, nor in the affected regions after the epidemic (*see de Vogel, 1939*). This close relationship between the occurrence of the El Tor vibrio and the presence of cholera in the Celebes has not been noticed elsewhere. The repeated finding of vibrio El Tor in open natural water sources in certain rural areas in Tanjore district, South India, in the complete absence of cholera in the region, during the entire period of observation and for several months previously, is therefore considered worthy of record.

* The term 'agglutinable vibrio' is used throughout to denote vibrios possessing the specific group I 'O' antigen of Gardner and Venkatraman (1935) and includes both the true 'cholerae' and the hæmolytic El Tor vibrio. All other vibrios are referred to as 'non agglutinable'.

The observations reported in this paper were made in the course of a survey of open natural water sources in the Cauvery Delta during 1940, and formed part of a larger investigation of the conditions of prevalence of true *Vibrio cholerae* in the local population and external sources. The region had been entirely free from cholera from May of the preceding year, 1939, and has continued to be so. The examination of 1,827 stool specimens from the inhabitants of the region failed to reveal the presence of agglutinable vibrios in any.

Previous investigations in this direction have been subject to the limitations of the technique available, which would not permit the recovery, with certainty, of agglutinable vibrios when present in small numbers in water or stools together with relatively more numerous non-agglutinable vibrios and other faecal organisms. The almost universal presence of non-agglutinable vibrios in waters in North India, under conditions in which cholera contamination could be excluded, has been reported by Taylor and Ahuja (1938). The experience of Pandit and Maitra (1938) in Assam has been the same. One of us (K. V. V.) has found similar conditions to obtain in the Madras Province.

METHODS

The modification of the bismuth-sulphite enrichment medium of Wilson and Blair described by Read (1939) has greatly helped to overcome this difficulty in the successful recovery of agglutinable vibrios, especially when the opposing non-agglutinable vibrios present are non-mannose fermenters. This, however, fails sometimes, in the presence of mannose-fermenting non-agglutinable vibrios. The technique adopted by us in the examination of water has been essentially the same as described by Read (*loc cit*), and in control experiments with artificially inoculated waters, has enabled us regularly to recover *V. cholerae* from 2,500 c.c. of water in which 10 to 100 viable vibrios had been introduced along with a considerably heavier inoculum of a non-mannose fermenting non-agglutinable vibrio and *Bact. aerogenes*. In the work here reported, 2,500 c.c. quantities of water were collected from each source, sufficient sea-salt added to make a 1 per cent concentration and the pH brought up to 9.2 by the addition of sufficient N/1 NaOH solution, at the spot of collection. These were then transported to the Laboratory generally within 3 to 4 hours (often within one hour) and filtered through Kieselguhr impregnated filter-paper, which, with the deposit, was then taken in 100-c.c. stoppered-bottles containing 60 c.c. of the mannose-bismuth-sulphite medium and incubated overnight. Platings were made on Aronson and agar media from the enriched cultures. Colonies picked from these plates were first tested on a slide with a suitably diluted cholera 'O' agglutinating serum and if found to be agglutinable, subcultured on agar and submitted to further tests including agglutination in the tube with monotype Inaba and Ogawa 'O' sera and hæmolysis with goat erythrocytes. In the earlier part of the work, a certain number of non-agglutinating vibrio colonies were also picked out to determine their biochemical type, but reference to these is omitted in the rest of the paper. A number of colonies were picked off each plate to see if more than one agglutinable type was present.

The hæmolytic tests were done according to Greig's method, using a 72-hour extract broth or 48-hour peptone-water culture of vibrios and 10 per cent washed goat-erythrocytes. A certain amount of variation in the hæmolytic power of the vibrios was noticed when the tests were repeated. Latterly, all vibrio strains have been submitted to the test using a 24-hour culture in Douglas' broth when more uniform results were obtained.

A simple chemical analysis of water was made of most of the specimens, including an estimation of the total solids, chlorides and oxidizable matter. This was done with the object of determining if the composition of the waters in which agglutinable vibrios were found, differed in any manner from that of other specimens in the same region. No significant difference was noticed.

Eight hundred and seventy-eight specimens of water from 237 sources were examined by the method described above. These sources include rivers and channels, tanks, ponds, and a few wells, and represent the greater part of the deltaic portion of the Tanjore district and portions of Trichinopoly and South Arcot districts. Only one or two specimens were collected from each source in a number of cases in the preliminary survey but in regions where agglutinable vibrios were recovered, repeated examinations were made of all the natural sources in the village and the neighbourhood.

FINDINGS

Agglutinable vibrios were isolated from 21 specimens representing 17 different sources. These were limited to two small areas—one near Negapatam and the other near Thruthuraipundi, at the tail end of the delta. The particulars regarding these isolations are shown in the Table.

It will be seen that agglutinable vibrios have been isolated at one time or another during the year, from 12 out of the 33 tanks in the two villages adjoining Negapatam and 5 out of the 28 in and around Thruthuraipundi. In only two instances (Nos 1 and 11) have these vibrios been isolated more than once from the same source—thrice in the course of 6 days from No 1 and thrice in 29 days from No 11. The failure to isolate these vibrios in second and subsequent examinations may be due either to their rapid elimination after introduction, or to the inadequacy of the technique which, sometimes, fails in the presence of other mannose fermenters.

The serological type of these 21 strains of agglutinable vibrios was determined by agglutination tests with monotype Inaba and Ogawa 'O' sera. Twelve were of the Ogawa type and 9 Inaba. All cultures derived from a number of colonies from the same plating were of one type. Where more than one isolation was made from the same source, the type of vibrio has remained the same throughout. The Inaba and Ogawa tanks are not disposed in any clear natural groups, but are scattered irregularly in the village.

The hæmolytic property of these vibrio strains was tested by methods referred to in a previous paragraph. Nineteen were frankly hæmolytic, one gave a trace of hæmolytic at the time of isolation and the other was non-hæmolytic. Differences were noticed in the degree of hæmolytic produced by cultures derived from several

Particulars of isolation of agglutinable

1940										
Source	FEBRUARY		MARCH		APRIL		MAY		JUNE	
	Number of examina- tions	Number of isolations	Number of examina- tions	Number of isolations	Number of examina- tions	Number of isolations	Number of examina- tions	Number of isolations	Number of examina- tions	Number of isolations
<i>Negapalam (Indanapettai and Karavelankudai)</i>										
1 Chatramkulam	6	3	2	0	4	0			2	0
2 Veeridiankulam	5	1	2	0	4	0			2	0
3 Pidarikulam	4	1	2	0	4	0			2	0
4 Vinaitthurthankulam	2	1	3	0	4	0			2	0
5 Kamakshikulam	2	1	4	0	4	0			2	0
6 Aiyankulam	4	1	2	0	4	0			2	0
7 Vettukulam (Andanapettai)	2	0	2	0	4	0			2	0
8 Poongulam	3	0			4	0			3	1
9 Vannankulam	2	0			4	1			2	0
10 Sambukulam	4	1	2	0	4	0			2	0
11 Vettukulam (Karavelankadai)	4	2	2	1	4	0			2	0
12 P Odaikuttai	2	0			4	1			2	0
21 Other sources	38	0	15	0	63	0			34	0
<i>Thiruthuraiyandi</i>										
13 Chekkadikulam					3*	1				
14 Thelikulam					3*	1				
15 Nandavanamkulam										
16 Aiyankulam										
17 School pond										
23 Other sources					21	0				

* The first examination was

LF

vibrios from water sources

1940										Type of vibrio		
JULY		AUGUST		SEPTEMBER		OCTOBER		NOVEMBER			DECEMBER	
Number of examinations	Number of isolations	Number of examinations	Number of isolations	Number of examinations	Number of isolations	Number of examinations	Number of isolations	Number of examinations	Number of isolations		Number of examinations	Number of isolations
		2	0	2	0			1	0	2	0	Inaba hæmolytic
		2	0	2	0			1	0	2	0	Inaba hæmolytic
		2	0	2	0			1	0	2	0	Inaba hæmolytic
		2	0	2	0			1	0	2	0	Ogawa hæmolytic
		2	0	2	0			1	0	2	0	Ogawa hæmolytic
		2	0	2	0			1	0	2	0	Inaba hæmolytic
		2	1	2	0			1	0	2	0	Inaba non hæmolytic
		2	0	2	0			1	0	2	0	Ogawa hæmolytic
		2	0	2	0			1	0	2	0	Ogawa hæmolytic
				2	0					2	0	Inaba hæmolytic
				2	0					2	0	Ogawa hæmolytic
				2	0					2	0	Inaba non hæmolytic
		26	0	38	0			10	0	25	0	
4	0											Ogawa hæmolytic
4	0											Ogawa hæmolytic
6	1											Ogawa hæmolytic
4	1											Ogawa hæmolytic
1	1											Ogawa hæmolytic
49	0											Ogawa hæmolytic

made on 30th March, 1940

made on 30th March, 1940

colonies from the same plating, especially when the tests were done with 48- or 72-hour peptone-water cultures. When the tests were repeated some months later, using a 24-hour Douglas' broth culture, more uniform results were obtained. All cultures derived from the same plating were either all hæmolytic or all non-hæmolytic. Thus, 19 strains derived from 15 sources were hæmolytic and the other two non-hæmolytic, and are shown in the last column of the Table.

These 19 strains, possessing as they do, the specific group I 'O'-antigen as determined by agglutination with specific sera, and being hæmolytic are to be considered as El Tor vibrios, the other two are indistinguishable from true *V. cholerae*.

Reference has already been made to the absence of cholera in the region and the uniformly negative results in the examination of 1,827 stool specimens from the inhabitants of the districts. During February 1940, when the hæmolytic agglutinable vibrio was isolated from as many as 8 tanks in one of the villages near Negapatam, a very careful local inquiry was instituted among the people residing in the neighbourhood of the positive tanks. Eight cases of mild diarrhoea were detected, from none of whom could agglutinable vibrios be recovered. Stools of 257 healthy persons of the locality were also examined with negative results. No association could, thus, be established between the finding of these vibrios in the water sources and human disease in the village.

SUMMARY

The isolation of hæmolytic agglutinable vibrios (vibrio El Tor) from 15 open natural water sources in two rural areas in the Tanjore district, in the absence of cholera, is recorded. Non-hæmolytic agglutinable vibrios indistinguishable from true *V. cholerae* were also recovered from two other sources in the same area.

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STATISTICAL INQUIRY INTO THE EPIDEMIOLOGY OF CHOLERA IN BENGAL

Part I

A GENERAL REVIEW OF THE EPIDEMIOLOGICAL FEATURES OF CHOLERA IN DIFFERENT PARTS OF BENGAL

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BENGAL has generally been recognized as an endemic home for cholera from which, according to Bryden and Cornish, it spread by the northern epidemic highway into Bihar, the United Provinces and the Punjab and, by the southern epidemic highway, through the Central Provinces, to the western parts of the Bombay Presidency, including Gujerat, and southwards into the Deccan and the Madras Presidency. Epidemics originating from Bengal are believed to have occasioned pandemics involving greater part of the inhabited globe. The epidemiology of cholera in India has, therefore, been the subject of study by several writers ever since the advent of the Europeans in this country. The work of the earlier investigators such as Bryden, MacNamara and Bellow was mainly descriptive. More recently, Sir Leonard Rogers (1928) and Russell and Sundararajan (1928) have studied the problem by quantitative analysis of data. All of them came to the conclusion that cholera was endemic in Bengal.

Bryden's endemic area included 'the western portion of Assam, all deltaic regions of lower Bengal and Orissa up to the low Rajmahal and Cuttack hills to the

west of this basin'. Eastern but not the western Bihar was included in his endemic area. His views regarding the spread of cholera from Bengal into other parts of India have already been stated.

The epidemiological studies of Russell and Sundararajan (*loc cit*) consisted of two parts, (i) an investigation into the periodicity of cholera in the different provinces of India and (ii) a study of the relationship of meteorological conditions to the incidence of the disease. They demonstrated that cholera had a six-yearly periodicity in western Bengal and a five-yearly cycle in eastern Bengal. Climatic factors, they found, had considerable influence on the incidence of the disease in various parts of India but they seemed to have no effect on the incidence in Bengal and Assam. For these reasons, they concluded, that the disease was endemic in these provinces.

Rogers' (*loc cit*) work also covered the whole of India. He stated that lower Bengal, Orissa and Assam constituted a hyperendemic area of cholera because the variation in its incidence from year to year was very small. Rogers observed that in spite of the increased facilities of communication by railways, the progress of cholera from Bengal towards the west and north still followed the time table sketched by Bryden. To explain this fact he advanced the view that for the spread of cholera a minimum of 0.4 mm pressure of absolute humidity was required. In Bengal the absolute humidity remains above the critical level all the year round but in the western provinces this level is reached only in certain parts of the year. As we proceed westward from Bihar to the Punjab the period of favourable humidity becomes shorter and shorter and is progressively delayed. These circumstances, according to him, accounted for the elimination of any influence which the development of communications might have had in accelerating the spread of the disease.

Recently, in a more detailed study Fry (1925) has followed the shift of the cholera peak to a later part of the year as one proceeds from east to west.

This brief survey will serve to show that the previous authors recognized an extensive area comprising of Assam, Bengal and Orissa as one endemic centre of cholera. From this endemic home the disease extended to other parts of the country but on account of unfavourable climatic conditions failed to establish itself permanently. The first question that presents itself is whether this vast extent of territory embracing approximately 146,000 square miles represents a homogeneous unit with regard to the natural history of cholera or whether it is composed of heterogeneous elements. The study of this problem is a pre-requisite to the necessary elucidation of some of the obscure points in the epidemiology of the disease.

The only available records on which statistical studies can be based are cholera mortality figures. The records of vital statistics in India are notoriously unreliable and the cholera case mortality is liable to variation. It is, therefore, recognized that these data cannot be expected to represent correctly the incidence of the disease. Since however, cholera, as a rule, presents well-marked symptoms, and the country people are familiar with it and its consequences, the mortality figures for cholera

may be reasonably considered as more reliable than they are for most other diseases. As a preliminary step we examined cholera mortality records from 1901 to 1930 for all the districts of Bengal. The mortality rates were calculated for the whole of the period as well as for the six quinquennial periods. They were based on mean populations for the respective periods as estimated by the geometrical progression method. The mean cholera rate for the whole period for the province as a whole was found to be 1.92 *per mille*. Using this rate for purposes of comparison the districts of Bengal could be divided into four groups as follows —

TABLE I

Distribution of the districts of Bengal into groups according to cholera mortality

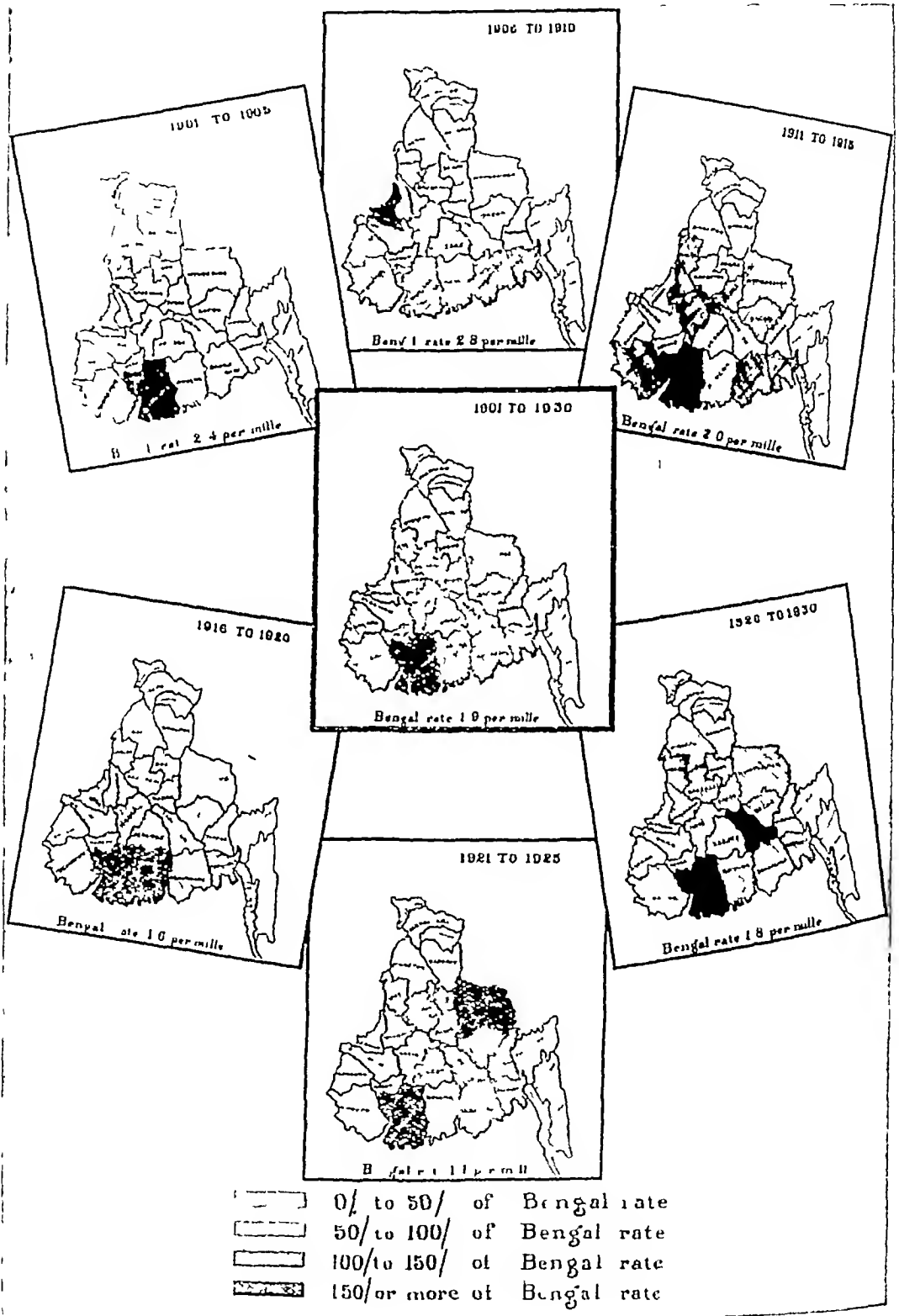
I	Low mortality rates ranging from 0 to 0.96 <i>per mille</i> , i.e. up to 50 <i>per cent</i> of Bengal rate Darjeeling, Jalpaiguri, Dinajpur
II	Moderate mortality rates ranging from 0.96 to 1.92 <i>per mille</i> , i.e. 50 <i>per cent</i> of Bengal rate to Bengal rate Rangpur, Bogra, Malda, Birbhum, Burdwan, Bankura, Hooghly, Tippera, Chittagong
III	High mortality rates ranging from 1.92 to 2.88 <i>per mille</i> , i.e. from Bengal rate to 50 <i>per cent</i> over Bengal rate Mymensingh, Pabna, Rajshahi, Murshidabad, Nadia, Jessore, Faridpur, Dacca, Noakhali, Backergunj, Khulna, Midnapore
IV	Very high mortality rates of 2.88 <i>per mille</i> or more 24-Parganas, Howrah

Mortality rates for the individual districts for the whole period and for the six quinquennial periods are given in *Appendix I*. Map 1 presents these data according to the above classification. From this rough analysis it will be readily seen that the districts exhibit considerable heterogeneity with regard to the degree of incidence of cholera and its variability.

Howrah and 24-Parganas stand out as the most severely affected districts. Next comes the central block of districts. The western group consisting of Bankura, Hooghly, Burdwan and Birbhum shows moderate incidence in spite of more extensive communication supplied to them by railways, roads and canals. The northern group, viz. Darjeeling, Jalpaiguri and Dinajpur, enjoys a comparative freedom from the disease.

MAP I.

MAP OF BENGAL SHOWING CHOLERA MORTALITY RATES PER MILLE FOR THE PERIOD 1901 TO 1930 AND FOR THE SIX INTERVENING QUINQUENNIAL PERIODS

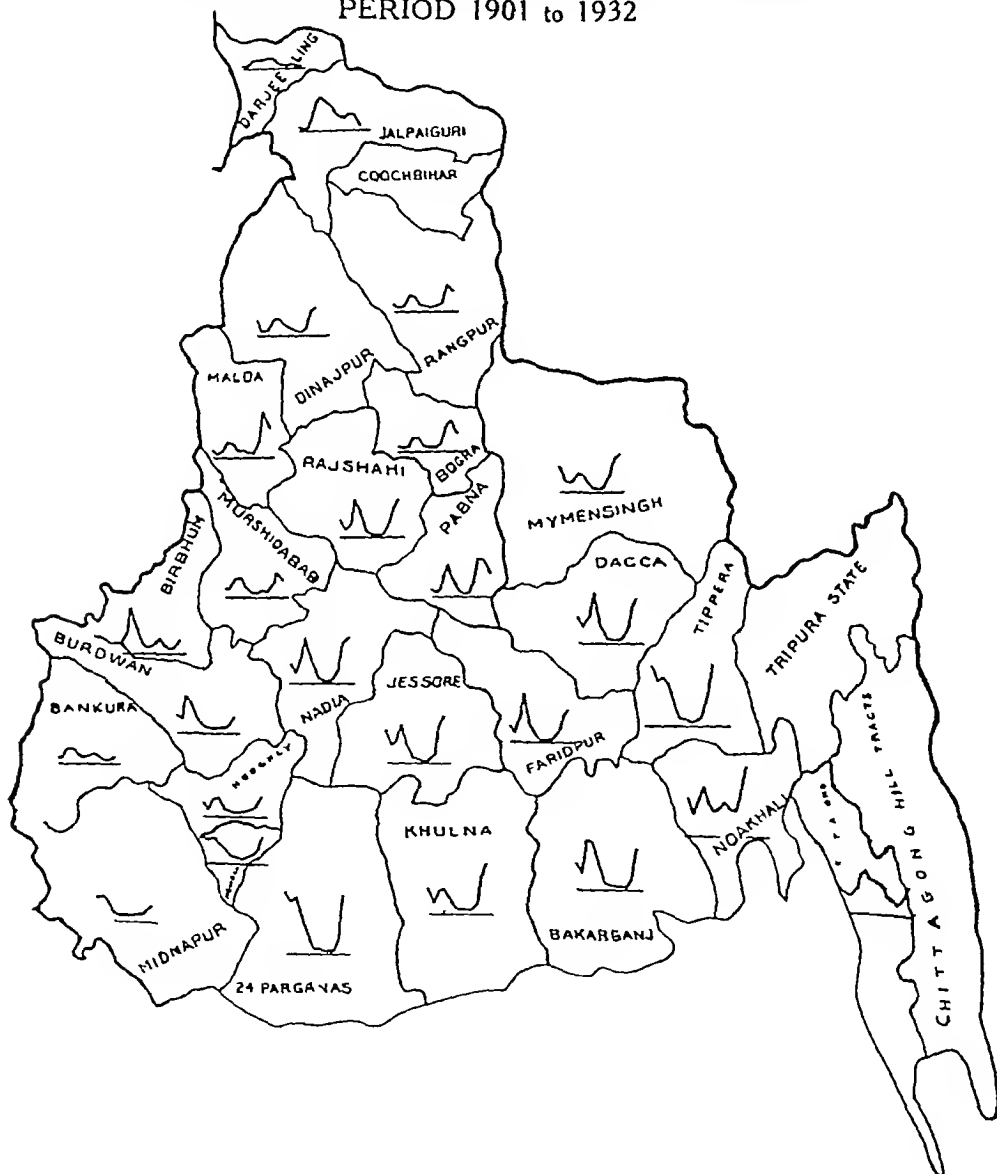


MEAN SEASONAL DISTRIBUTION OF CHOLERA MORTALITY IN THE DISTRICTS

Seasonal curves separately for each district are shown in Map 2

MAP 2

SEASONAL DISTRIBUTION OF MEAN CHOLERA MORTALITY
IN THE DISTRICTS OF BENGAL FOR THE
PERIOD 1901 to 1932



Seasons in Bengal may be divided as follows —

- (1) *Spring-summer* — Hot and dry and extends from the middle of February to the middle of June
- (2) *Rainy season* — Hot and wet from the middle of June to the middle of October
- (3) *Autumn-winter* — Cold and dry from middle of October to the middle of February

With regard to the seasonal incidence the various districts in Bengal may be roughly divided into three groups —

- (1) Bimodal with one peak in autumn-winter and the other in spring-summer, the two being separated by a dip in February. The main trough corresponds to the monsoon months. Majority of the districts belong to this group
- (2) A unimodal curve in which the dip in February is practically eliminated. 24-Parganas belongs to this group
- (3) Bankura, Darjeeling and Birbhum differ from other districts in that they exhibit an evidence of a rise in the rainy season

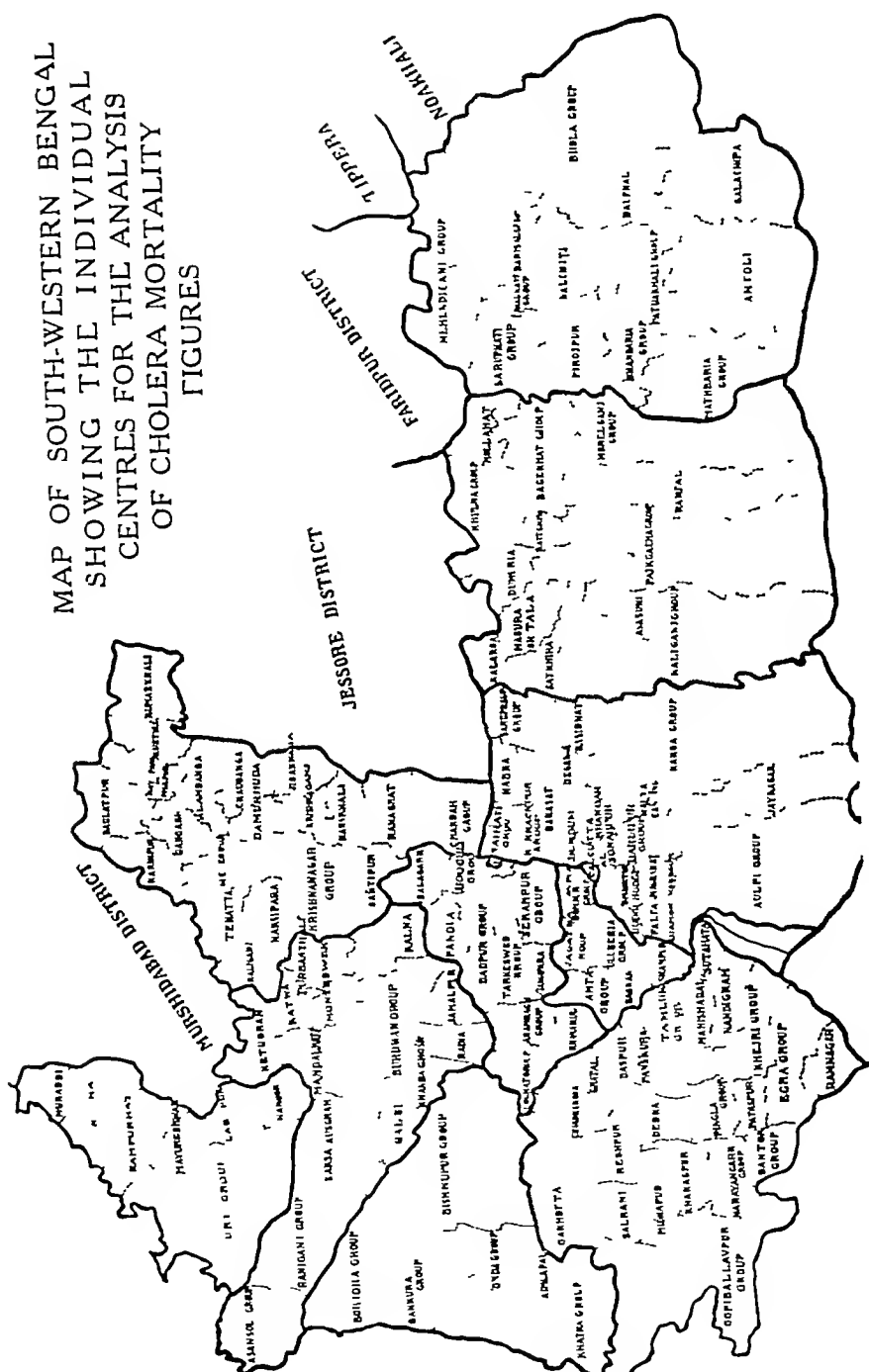
This gross analysis of cholera mortality figures by districts clearly shows that Bengal is far from being a homogeneous area in regard to its cholera experience. For the purpose of study of the natural history of the disease it becomes necessary to divide it into homogeneous areas. Many of the districts, however, are separated from each other by artificial boundaries which have been fixed for purpose of administrative convenience. These units, therefore, may not represent areas of homogeneous cholera experiences. This point is clearly illustrated by the contiguous districts of Hooghly and Howrah which exhibit marked differences in the cholera incidence. In order, therefore, to demarcate homogeneous cholera districts it is necessary to take the smallest geographical unit for purpose of investigation. The *thanas* or the police circles which have for a long time been the registration centres in Bengal and for which cholera mortality figures are separately available for extended periods provide the most suitable units for this purpose*.

CHOLERA MORTALITY BY *thanas*

The adoption of *thanas* as the units for study was, however, not free from difficulties. During the period under investigation many of the *thanas* underwent changes as the result of a re-distribution of the areas under their jurisdiction for administrative or other reasons. Some *thanas* disappeared as distinct units, having been merged in whole or in part in one or more of the adjacent *thanas*, while on other occasions new *thanas* were created. Exact knowledge of these changes is necessary for relating cholera deaths to the corresponding populations. When these changes took place during the intercensal periods and when, as it happened in some cases,

* The data on which the discussion is based in this communication and in Part II of this series relate to rural areas only.

MAP OF SOUTH-WESTERN BENGAL
SHOWING THE INDIVIDUAL
CENTRES FOR THE ANALYSIS
OF CHOLERA MORTALITY
FIGURES



the newly constituted *thanas* did not become registration centres for sometime afterwards or failed to do so altogether, the adjustment of the populations to the recorded deaths became wellnigh impossible. Every care was exercised to make these adjustments wherever possible and for this purpose the government notifications kindly supplied to us by the Inspector-General of Police, Bengal, were freely utilized. In case, however, where the information required was not available the units were taken as they existed at the census enumeration of 1911. In complicated cases a number of *thanas* concerned was combined and treated as single units. Map 3 and *Appendix II* show the centres which have been treated as units for the purpose of this study.

Since this detailed investigation involved considerable amount of work the study was confined to a group of 10 districts, namely, Backergunj, Khulna, 24-Parganas, Nadia, Birbhum, Burdwan, Bankura, Midnapore, Hooghly and Howrah.

HETEROGENEITY WITHIN THE DISTRICTS

The range of variation in the mean cholera mortality rates for the whole period between the various *thanas* in each district is presented in Table II —

TABLE II

The highest and the lowest mean cholera incidence for the whole period in thanas comprising the various districts

Number	Name of districts	MEAN CHOLERA MORTALITY RATES PER 10,000 IN <i>thanas</i>		
		Highest	Lowest	Difference
1	24 Parganas	6.61	1.32	5.29
2	Midnapore	5.22	0.56	4.66
3	Khulna	5.06	1.00	3.07
4	Howrah	5.13	2.70	2.43
5	Burdwan	3.55	1.36	2.19
6	Backergunj	3.46	1.88	1.58
7	Nadia	3.60	2.13	1.47
8	Hooghly	2.56	1.16	1.40
9	Bankura	2.29	0.98	1.31
10	Birbhum	2.63	1.81	0.82

In the table the districts have been arranged in order of the values of differences between the *thanas* showing the highest and the lowest incidence. Judged by this criterion 24-Parganas shows the greatest heterogeneity and Birbhum the least.

THE SEASONAL DISTRIBUTION OF CHOLERA MORTALITY IN *thanas*

The seasonal curves for *thanas* are presented in Map 4. An examination of these curves brings out the following points —

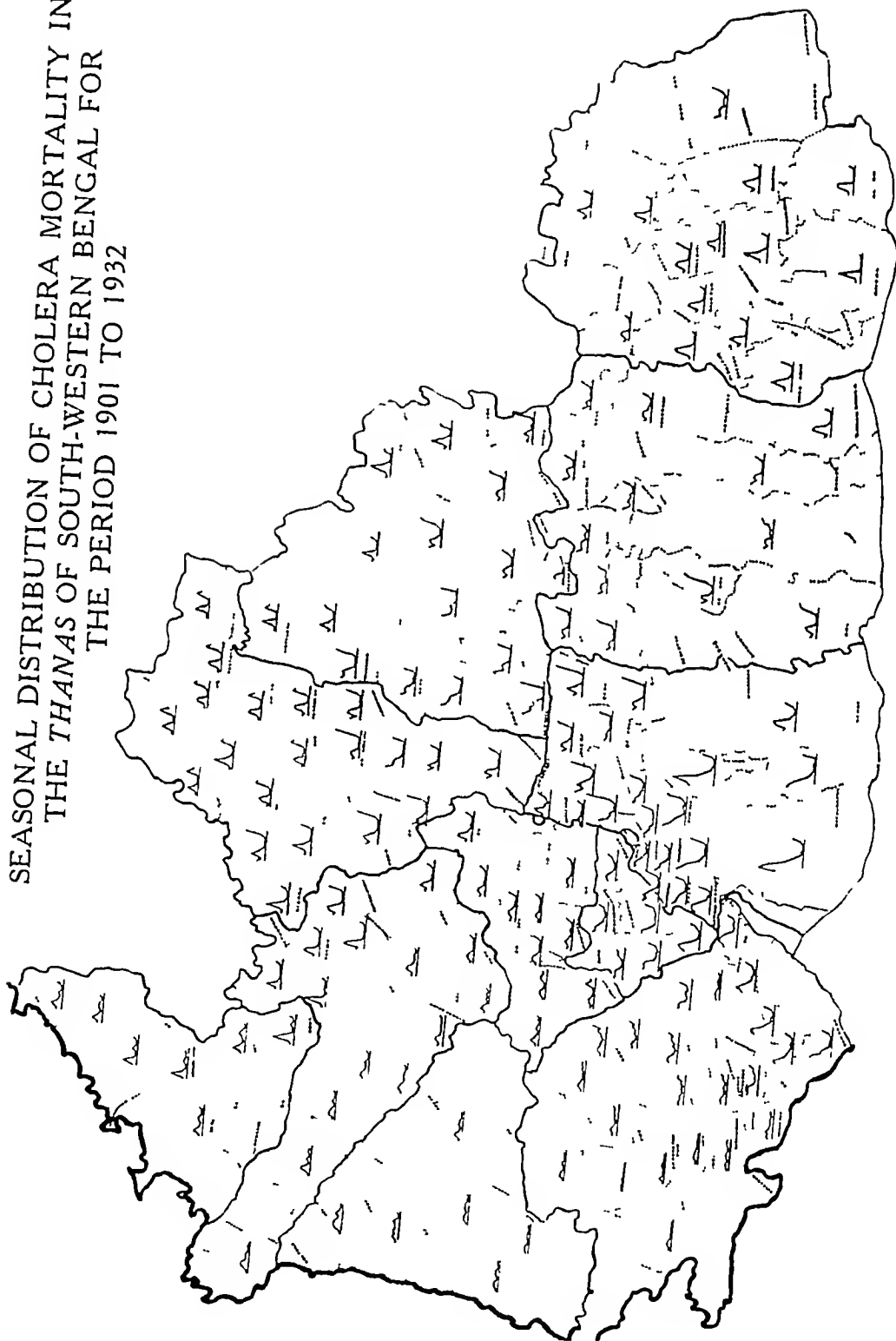
1 The south-western portion of the area under investigation has, relatively to the remaining portions, a much lower incidence.

2 The *thanas* exhibit among themselves considerable heterogeneity in regard to seasonal distribution of the disease. However, certain groups of contiguous *thanas* exhibit striking similarity. The whole area may be divided into seven broad groups based on similarity of shape of the curves of contiguous *thanas* —

- (a) The whole of Backergunj district except Bhola group. In this group Morelgunj group of Khulna district is also included. The maximum incidence is in spring-summer. Prevalence of cholera in autumn-winter is relatively low.
- (b) The remaining portion of Khulna district and the southern halves of Jessore and Nadia districts. Here, the winter incidence is higher than in the previous group and the rise during the spring-summer is not so prominent.
- (c) Canning (Matla) and Sandeshkhali group. This resembles the Backergunj group.
- (d) A group of centres on either side of the Hooghly in the districts of 24-Parganas, Howrah and Midnapore. The curve is unimodal and cholera is prevalent mainly in autumn-winter.
- (e) A big group consisting of Birbhum, the western part of Burdwan, the whole of Bankura, western portion of Hooghly and the whole of Midnapore with the exception of the centres included in the previous group. A special feature of this group is a small rise in cholera incidence during the rains. The total incidence is, on the whole, very low except in Birbhum.
- (f) A small group to the north-east of the previous one. It consists of the remaining *thanas* of Burdwan district and three contiguous *thanas* of Nadia district. Here the curve is bimodal with a high peak in spring-summer and a low one in winter-autumn.
- (g) Another group consisting of the northern portions of Nadia and Jessore districts. Both peaks are equally high. However, the autumn-winter peak occurs in November instead of in December as is usually the case.

MAP 4.

SEASONAL DISTRIBUTION OF CHOLERA MORTALITY IN
THE THANAS OF SOUTH-WESTERN BENGAL FOR
THE PERIOD 1901 TO 1932



3 From this description it will be observed that widely separated groups such as the one in the north-east of Burdwan district, Sandeshkhali-Canning (Matla) group in 24-Parganas and the group of *thanas* in Backergunj district are alike in seasonal distribution of cholera. The intervening areas present quite dissimilar experience.

4 It may also be seen from Table III that various units showing similarity in regard to seasonal incidence may have different mean rates —

TABLE III

Range of variation in mean incidence within an area showing similar seasonal incidence

	Minimum rate per 10 000	Maximum rate per 10,000	Difference
Group to the north east of Burdwan district	3.2	3.5	0.3
Sandeshkhali Canning area	4.0	5.7	1.7
Backergunj area	1.9	3.3	1.4

5 It will be remembered that with regard to the seasonal experience of cholera Fry contrasted the experience in Bengal with that of Bihar. In the former cholera was at its lowest during the monsoon season when in Bihar it was at its highest. It is interesting to note that *thanas* in group (e) referred to above constitute the area where transition occurs.

SIMILARITY BETWEEN CONTIGUOUS *thanas* OF DIFFERENT DISTRICTS

Table IV illustrates some instances in which there is similarity with regard to mean cholera mortality between contiguous *thanas* of two districts. It is apparent from the table that the lines dividing the districts run through areas having similar mean experience. The same point is brought out by the classification of the area into seven groups according to types of seasonal curves.

TABLE IV

Illustrating similarity between contiguous thanas of the different districts

Districts.	Centres	Cholera mortality rate per 10,000
Burdwan	{ Ketugram, Katwa, Purbasthali.	}
Nadia		
	Kalgunj, Nakshipara	ranges from 3.1 to 3.4

TABLE IV—*concl'd*

Districts	Centres	Cholera mortality rate per 10,000
Hooghly	Khanakul	2.7
Howrah	Amta group	2.2
Midnapore	Daspur	2.3
Midnapore	Garbotta	0.81
Bankura	Samlapal	0.99
Khulna	Satkhira	3.0
24 Parganas	Basirhat	3.1

SUMMARY

1 Previous writers on the epidemiology of cholera in India have recognized Bengal, Orissa and Assam as constituting one big endemic home of cholera

2 It has been shown that the districts of Bengal present considerable heterogeneity in regard to their cholera experience and that there are evidences of heterogeneity within the districts themselves

3 The necessity of undertaking an inquiry with a view to forming homogeneous cholera districts in connection with the study of the natural history of the disease has been discussed

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APPENDIX I

Cholera mortality in Bengal by districts for the period 1901 to 1930 and during the periods 1901-1905, 1906-1910, 1911-1915, 1916-1920, 1921-1925 and 1926-1930

Name of district	Rate per mille, 1901-30	Rate per mille, 1901-05	Rate per mille, 1906-10	Rate per mille, 1911-15	Rate per mille, 1916-20	Rate per mille, 1921-26	Rate per mille, 1926-30
1 Burdwan	1 71	1 81	3 49	1 46	1 90	0 67	1 22
2 Birbhum	1 78	1 32	4 30	1 50	2 24	0 57	0 94
3 Bankura	1 12	1 15	2 10	1 42	0 96	0 57	0 58
4 Midnapur	2 21	2 87	3 59	2 13	2 32	1 03	1 44
5 Hooghly	1 60	2 19	2 23	1 73	1 51	0 63	1 23
6 Howrah	2 97	4 02	3 84	3 39	2 53	1 39	2 85
7 24 Parganas	3 32	4 23	3 71	3 58	3 36	1 78	3 16
8 Nadia	2 49	3 46	3 87	2 46	1 92	0 96	2 29
9 Murshidabad	2 00	2 44	2 86	2 46	2 09	0 54	1 94
10 Jessore	2 39	3 05	3 45	1 95	1 74	1 52	2 60
11 Khulna	2 24	2 40	3 44	1 85	2 50	0 83	2 66
12 Rajshahi	2 14	2 47	4 03	1 52	1 00	1 37	2 23
13 Dinajpur	0 65	0 58	1 10	0 61	0 58	0 25	0 71
14 Jalpaiguri	0 85	0 29	1 42	0 96	1 39	0 64	0 25
15 Darjeeling	0 39	0 21	0 38	0 71	1 00	0 02	0 10
16 Rangpur	1 16	0 74	2 07	1 19	0 77	0 96	1 07
17 Bogra	1 41	2 34	2 18	1 08	0 72	1 07	1 00
18 Pabna	2 13	3 25	2 52	2 28	1 60	1 14	2 08
19 Malda	1 86	2 80	1 90	2 74	0 98	0 24	2 25
20 Dacca	2 43	3 04	2 44	2 71	1 33	1 52	2 21
21 Mymensingh	2 21	3 11	2 67	2 20	1 55	1 70	1 89
22 Fardpur	2 09	2 83	2 62	1 93	1 36	0 87	3 05

APPENDIX I—concl'd

Name of district	Rate per mille, 1901-10	Rate per mille, 1901-05	Rate per mille, 1906-10	Rate per mille, 1911-15	Rate per mille, 1916-20	Rate per mille, 1921-26	Rate per mille, 1926-30
23 Backergunj	2 00	2 04	3 83	2 30	1 23	1 06	1 72
24 Chittagong*	1 23	1 20	1 71	1 44	1 60	0 39	1 06
25 Noakhali	2 11	2 12	2 78	2 86	2 16	1 11	1 98
26 Tippera	1 67	1 02	1 98	1 67	1 45	1 26	1 74
Bengal Province excluding Calcutta	1 02	2 10	2 75	1 98	1 58	1 06	1 79
Calcutta	1 92	2 23	2 74	1 80	1 82	1 16	1 86

* Note.—The cholera figures for Chittagong Hill Tracts were not available, hence the population for this area has been excluded from Chittagong district and the total population for Bengal

APPENDIX II

List of centres which form units for investigation In the *brackets* are given the names of all the *thanas* which comprise one group

Birbhum district

Muraro, Nalhati, Rampurhat, Mayureswar, Labhpur, Nanoor, Suri group (Suri, Illambazar, Bolpur, Dubrajpur, Khoyrasole, Rajnagar, Muhammadbazar)

Burdwan district

Ketugram, Katwa, Purbasthali, Monteswar, Mangalkote, Burdwan group (Burdwan, Satgachia, Memari, Bhatar, Sahibganj), Kalna, Jamalpur, Rama, Khandaghosh, Galsi, Ausgram, Kaksa, Asansol group (Asansol, Kulti, Salampur, Barabani), Raniganj group (Raniganj, Jamuria, Ondal, Faridpur)

Bankura district

Borjora group (Borjora, Saltora, Mejhia, Gangajalghati), Bishnupur group (Bishnupur, Sonamukhi, Patrasar, Indas, Kotalpur, Jaipur, Radhanagar), Onda group (Onda, Taldangra), Khatra group (Khatra, Indpur, Raipur, Ramibandh), Bankura group (Bankura, Chatna)

APPENDIX II—concl'd

Midnapur district

Garhbeta, Chandrakona, Ghatal, Keshpur, Daspur, Panskura, Debra, Salbani, Midnapur, Kharagpur, Gopibullavpur group (Gopibullavpur, Jhargram, Jamboni, Binpur, Navagram), Egra group (Egra, Contai), Ramnagar, Khejri group (Khejri, Bhagwanpur), Nandigram, Satahata, Mahisadal, Tamluk group (Tamluk, Moyna), Pataspur, Pingla group (Pingla, Sabang)

Hooghly district

Goghat group (Goghat, Badanganj), Khanakul, Arambagh group (Arambagh, Pursoora), Hooghly group (Hooghly, Chinsura, Magra), Pandua, Tarakeswar group (Tarakeswar, Haripal), Serampur group (Serampur, Singur, Chanditala, Uttarpur), Dadpur group (Dadpur, Polba, Dhamakhali), Jangipara or Krishnanagar, Balgarh

Howrah district

Shampur group (Shampur, Mangalghat), Uluberia group (Uluberia, Bowria), Bagnan, Jagatballavpur group (Jagatballavpur, Panchla), Amta group (Amta, Singhi), Domejur group (Domejur, Sankrail, Bally, Lalooah)

24-Parganas district

Kulpi group (Kulpi, Kakdwip, Sagar, Mathurapur), Matla or Canning, Jaynagar, Baruipur group (Baruipur, Pratapnagar), Barasat group (Barasat, Amdanga, Rajarhat), Sarupnagar group (Sarupnagar, Baduria), Basirhat, Magrahat, Diamond Harbour, Haroa group (Haroa, Sandeshkhali, Hasnabad), Bishnupur, Sonerpur, Falta, Budge-Budge, Naihati group (Naihati, Jagatdal, Bijpur), Dum Dum, Titagarh group (Titagarh, Barrackpore, Noapara, Khardah), Bhangar, Habra, Deganga

Khulna district

Dumuria, Batiaghata, Paikgacha group (Paikgacha, Dacope), Kalaroa, Magura or Tala, Satkhira, Khulna group (Khulna, Tarakhuda, Daulatpur, Palarhat, Fultola), Kalgunj group (Kalgunj, Shyamnagar, Debhata), Asasuni, Bagerhat group (Bagerhat, Kachua, Fakirhat), Rampal, Morelganj group (Morelganj, Sarankhola), Mollahat

Backergunj district

Nalchiti, Backergunj, Pirojpur group (Pirojpur, Kowkhali), Sarupkati group (Sarupkati, Nazirpur, Binaripara), Mathbaria group (Mathbaria, Patharghata, Bamna), Bhandaria group (Bhandaria, Kathaha), Patuakhali group (Patuakhali, Betagi, Mirzapur), Bauphal, Amtoli group (Amtoli, Barguna), Galachipa, Barisal group (Barisal, Babuganj), Mehendiganj group (Mehendiganj, Gaurnadi, Badartuni, Muladi, Uzirpur), Jhalakati group (Jhalakati, Rajapur), Bhola group (Bhola, Daulatkhan, Baranadi, Tazmuddin, Lahmohan)

Nadia district

Chuadanga, Damurhuda, Alamdanga, Jibannagar, Kaliganj, Krishnagar group (Krishnagar, Chapra, Nadia or Nabadwip), Nakasipara, Krishnaganj, Hanskhali, Santipur, Ranaghat, Kusthia, Mirpur or Nayapara, Kumarkhali group (Kumarkhali, Khoksha), Daulatpur, Karimpur, Gangani, Meherpur, Tehatta, Chakdah group (Chakdah, Haringhata)

STATISTICAL INQUIRY INTO THE EPIDEMIOLOGY OF CHOLERA IN BENGAL

Part II.

FORMATION OF HOMOGENEOUS CHOLERA DISTRICTS

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IN a previous communication (Lal *et al*, 1941) it was shown that there was considerable heterogeneity among the districts with regard to cholera incidence as well as among the *thanas* constituting individual districts. It was further pointed out that a distribution of the *thanas* into homogeneous cholera districts was the essential pre-requisite for a systematic study of the epidemiology of cholera and for the formulation of suitable methods of forecast. It is, therefore, necessary to devise methods to secure adequate description of the characteristics of each *thana* in numerical terms. The next step would be to determine the extent of similarity in respect to these characteristics in contiguous *thanas*.

NUMERICAL PRESENTATION OF THE EPIDEMIOLOGICAL CHARACTERISTICS OF A LOCALITY

Two criteria that appear to be suitable for giving quantitative expression to the cholera experience of a locality are (1) the mean incidence and (2) the variability

of incidence round the mean. Variations in mean incidence have been briefly discussed in the preceding paper. In regard to the second criterion, cholera presents, like many other infectious diseases, marked seasonal and yearly variations. The separation of these two types of fluctuation from the total variability appeared to be essential for describing the epidemiology of the disease. The material for our study consisted of the monthly mortality figures during the period of 32 years, 1901-32. In order to eliminate the variations due to the changes in population during this period, the monthly mortality figures of each *thana* were converted into rates per 10,000 of population. Interpolation by arithmetical progression was used to estimate the mid-year population for each year.

For separating the seasonal and yearly variations in cholera mortality Fisher's (1936) method of analysis of variance was used. A description of the method and a discussion of its applicability to problems such as this have been given in another communication (Raja *et al*, 1938).

This method divides the total variability into three parts. (1) Seasonal variability or between months—this provides a numerical measure of the mean variation in cholera incidence due to climatic factors or other factors linked up with seasons such as movements of population caused by fairs, festivals and other socio-economic causes and also dietetic changes. (2) Variability between years—this is a measure of epidemicity. (3) Residual variability—this includes variations due to all other causes.

For convenience the procedure followed is summarized in Table I —

TABLE I
Analysis of variance

Source of variation	Degrees of freedom	Sum of squares	Mean square	Contribution to total variance
Months	11	$S (\bar{x}_u - \bar{x})^2$	$\frac{1}{11} S (\bar{x}_u - \bar{x})^2 = P_1$	$\frac{P_1 - P_3}{32}$
Years	31	$S (\bar{x}_v - \bar{x})^2$	$\frac{1}{31} \times S (\bar{x}_v - \bar{x})^2 = P_2$	$\frac{P_2 - P_3}{12}$
Residual	341	$S (x_{uv} - \bar{x}_u - \bar{x}_v + \bar{x})^2$	$\frac{1}{341} S (x_{uv} - \bar{x}_v - \bar{x}_u + \bar{x})^2 = P_3$	$= P_3$
TOTAL	383	$S (x_{uv} - \bar{x})^2$		

where S represents summation over all the 384 monthly values (monthly cholera mortality over the period 1901-32), x_{uv} is an individual monthly value, \bar{x}_u the mean of the 32 yearly values for the u th month, \bar{x}_v the mean of the 12 monthly values for the same year and \bar{x} the mean of the 384 observations. If P_1 and P_2 are significantly greater than P_3 as shown by Z-test then a true estimate of monthly variance is $(P_1 - P_3)/32$ and of yearly variance $(P_2 - P_3)/12$. These values together with P_3 and the means are given in Table II (*vide* Map 1).

TABLE II

Means* and the monthly, yearly and residual variances of the thanas

Number	DISTRICT	MEAN	CONTRIBUTION TO		Residual variance
	Thana	Average of the 384 mortality rates per 10,000 population.	Monthly variance	Yearly variance	
	<i>Birbhum district</i>				
1	Mayureswar	2.55	5.739	11.716	78.467
2	Nanoor	2.63	9.909	7.931	94.262
3	Rampurhat	2.43	5.015	11.656	83.315
4	Nalhati	2.32	1.060	6.819	42.318
5	Suri group	1.81	1.285	8.521	18.145
6	Labhpur	2.08	1.759	9.062	41.079
7	Muraro	2.08	1.614	4.614	25.609
	<i>Burdwan district</i>				
1	Khandaghosh	1.63	0.643	1.808	11.754
2	Ausgram	1.58	0.678	2.863	11.569
3	Burdwan group	1.93	3.499	1.835	8.630
4	Galsi	1.69	0.837	2.421	9.578
5	Kaksa	1.36	0.819	2.170	8.772
6	Monteswar	3.22	14.651	15.322	60.708
7	Katwa	3.21	13.520	9.535	62.718

* The term 'mean' has been adopted to represent the average of the 384 mortality rates per 10,000 population.

*Epidemiology of Cholera in Bengal*TABLE II—*contd*

TABLE II—*Contd.*

Number	DISTRICT	MEAN	CONTRIBUTION TO		Residual variance.
	<i>Thana</i>	Average of the 384 mortality rates per 10,000 population	Monthly variance	Yearly variance	
<i>Burdwan district—concl'd</i>					
8	Purbasthali	3 38	10 646	7 663	45 441
9	Mungalkote	3 55	19 520	10 564	88 498
10	Ketugram	3 13	14 481	5 772	62 009
11	Kalna	2 44	4 429	2 426	20 368
12	Asansol group	1 69	1 995	5 606	29 210
13	Ranigunj group	1 60	1 012	5 931	21 320
14	Jamalpur	1 45	0 712	0 923	7 224
15	Rama	1 65	0 679	1 485	5 889
<i>Bankura district</i>					
1	Simlapal	0 99	0 262	0 535	6 564
2	Onda, Taldangra	0 98	0 080	0 711	2 330
3	Borjora group	1 71	1 212	4 037	18 035
4	Khatra group	2 20	1 629	20 169	11 679
5	Bankura group	1 45	0 331	2 168	7 084
6	Bishnupur group	1 35	0 668	1 032	2 945
<i>Midnapur district</i>					
1	Maslandpur (Maisadal)	4 70	9 367	7 664	18 717
2	Egra and Contai	1 34	14 932	5 940	23 634
3	Narayangarh Keshuery	1 63	0 148	1 473	5 875
4	Nandigram	5 08	11 179	9 679	19 676
5	Tamluk and Moyna	4 00	6 782	6 395	7 518
6	Ramnagar	3	17 11	53	87 240
7	Bhagwanpur and Khejri		16 17	2	29 100

TABLE II—*contd*

Number	DISTRICT	MEAN	CONTRIBUTION TO		Residual variance
	Thana	Average of the 384 mortality rates per 10,000 population	Monthly variance	Yearly variance	
Midnapur district—concd.					
8	Midnapur rural	1 10	0 217	0 820	6 151
9	Debra	1 97	0 535	1 160	5 285
10	Keshpur	1 87	0 477	1 751	9 703
11	Salbani	0 52	0 024	0 155	1 146
12	Garhbetta	0 81	0 113	0 304	2 775
13	Daspur	2 30	1 601	1 885	8 323
14	Danton and Mohanpur	2 85	0 304	5 886	15 105
15	Pingla and Sabang	2 46	1 214	1 411	6 960
16	Kharagpur	0 86	0 000	0 427	1 751
17	Chandrakona	1 25	0 010	0 051	0 707
18	Ghatal	2 12	0 865	3 253	21 700
19	Panskura	3 01	2 132	3 166	9 251
20	Gopiballavpur group	0 56	0 025	0 237	0 964
21	Sutahata	4 50	6 417	5 643	24 576
22	Pataspur	4 23	4 295	6 794	85 926
24-Parganas					
1	Falta	3 67	8 890	4 203	25 814
2	Diamond Harbour	4 06	15 551	4 751	33 576
3	Deganga	1 96	4 109	1 082	27 233
4	Magrahat	5 11	33 684	11 298	74 607
5	Bishnupur	3 36	7 842	2 046	17 639
6	Bhangar	3 03	4 976	2 354	22 767
7	Haroa group	4 00	12 273	6 503	18 315
8	Habra	2 33	6 014	1 204	18 604

TABLE II—*contd*

TABLE II—contd

Number	DISTRICT	MEAN	CONTRIBUTION TO		Residual variance
	Thana	Average of the 384 mortality rates per 10,000 population	Monthly variance	Yearly variance	
<i>Burdwan district—concl'd</i>					
8	Purbasthali	3 38	10 646	7 663	45 441
9	Mangalkote	3 55	19 520	10 564	88 498
10	Ketugram	3 13	14 481	5 772	62 009
11	Kaln	2 44	4 429	2 426	20 368
12	Asansol group	1 69	1 995	5 606	29 210
13	Ranigunj group	1 60	1 012	5 931	21 320
14	Jamalpur	1 45	0 712	0 923	7 224
15	Rana	1 65	0 679	1 485	5 889
<i>Bankura district</i>					
1	Simlapal	0 99	0 262	0 535	6 364
2	Onda, Taldangra	0 98	0 080	0 711	2 330
3	Borjora group	1 71	1 212	4 037	18 035
4	Khatra group	2 29	1 629	20 169	11 679
5	Bankura group	1 45	0 331	2 168	7 084
6	Bishnupur group	1 35	0 668	1 032	2 945
<i>Midnapur district</i>					
1	Maslandpur (Maisadal)	4 70	9 367	7 664	48 717
2	Egra and Contai	4 34	14 932	5 940	23 634
3	Narayangarh Keshuary	1 63	0 148	1 473	5 875
4	Nandigram	5 08	11 179	9 679	19 676
5	Tamluk and Moyna	4 00	6 782	6 395	7 548
6	Ramnagar	4 97	17 111	9 053	87 240
7	Bhagwanpur and Khejri	5 22	16 175	9 303	29 100

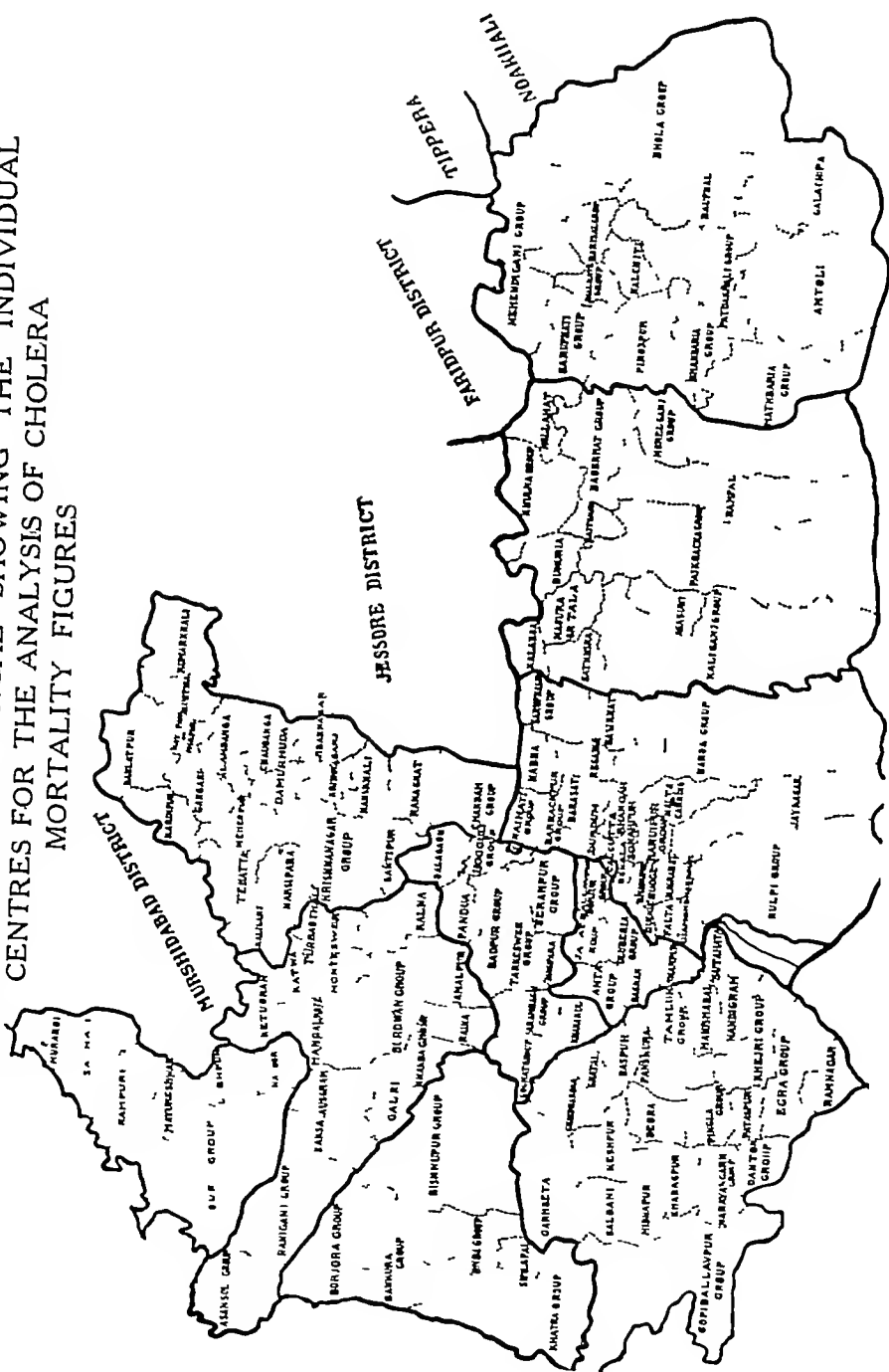
TABLE II—*contd*

Number	DISTRICT	MEAN	CONTRIBUTION TO		Residual variance
	<i>Thana</i>	Average of the 384 mortality rates per 10,000 population	Monthly variance	Yearly variance	
	<i>Hooghly district</i>				
1	Arambagh and Pursoora	1 78	0 408	1 713	8 780
2	Krishnagar (Jangipara)	1 16	0 529	0 166	2 261
3	Dadpur group	1 81	1 766	1 181	6 261
4	Serampur group	1 64	8 118	0 829	2 613
5	Hooghly and Magra	2 11	2 871	1 059	15 662
6	Tarakeswar and Haripal	1 76	1 233	0 758	6 148
7	Pandua	1 63	1 410	0 443	6 240
8	Khanakul	2 23	1 119	2 343	19 008
9	Balgarh	2 56	5 777	2 665	20 073
10	Goghat group	1 42	0 709	1 131	6 134
	<i>Howrah district</i>				
1	Shampur	5 13	14 548	7 111	25 536
2	Uluberia and Bowrin	4 34	8 407	4 469	18 721
3	Amta and Singli	2 70	1 427	1 919	11 997
4	Domjur group	3 13	2 592	2 005	8 197
5	Jagatballavpur group	3 29	4 187	2 758	16 973
6	Bagnan	4 19	6 990	3 785	16 620
	<i>Backerganj district</i>				
1	Nalchiti	2 99	9 988	2 485	19 313
2	Backerganj	2 18	5 822	1 435	12 969
3	Pirojpur	2 52	10 238	6 404	29 358
4	Sarupkati	1 88	3 330	1 372	8 229
5	Mathbaria	3 46	12 556	9 813	57 919
6	Bhandara	2 91	10 499	6 425	29 781
7	Patuakhali	3 27	18 625	3 548	31 241
8	Bauphal	2 70	5 688	1 670	9 493
9	Amtoli	2 44	13 098	3 371	34 364
10	Golachipa	3 02	13 031	4 653	23 461
11	Barisal	2 71	9 897	1 794	24 758

TABLE II—*concl'd*

Number	DISTRICT	MEAN	CONTRIBUTION TO		Residual variance
	Thana	Average of the 384 mortality rates per 10,000 population	Monthly variance	Yearly variance	
Backerganj district—concl'd					
12	Mehendiganj	2 29	4 366	0 716	9 092
13	Jhalakati	2 82	9 712	3 126	23 989
14	Bhola	3 25	5 863	5 054	13 972
Nadua district					
1	Nayapara	2 69	7 044	4 718	33 475
2	Damurhuda	3 36	9 015	7 673	82 863
3	Chuadanga	3 38	8 009	5 678	63 429
4	Ganganj	2 45	6 303	4 540	91 564
5	Krishnaganj	3 25	14 843	1 152	80 982
6	Chapra and Krishnagar	3 58	13 365	4 835	57 996
7	Santipur	3 60	13 832	4 142	51 538
8	Nakasipara	3 26	11 508	5 610	65 069
9	Tehatta	3 32	12 160	9 107	44 997
10	Kalganj	3 34	13 301	7 792	79 629
11	Ranaghat	2 99	9 089	3 011	31 949
12	Karimpur	2 13	5 169	5 253	42 726
13	Daulatpur	3 04	10 275	8 718	80 497
14	Alamdanga	2 80	8 066	3 681	57 664
15	Kumarkhali	2 77	9 121	3 608	58 262
16	Meherpur	3 40	7 384	7 917	111 660
17	Chakdaha	2 94	9 438	8 269	32 451
18	Kusthia	3 21	10 429	2 676	44 225
19	Hanskhali	3 27	13 357	4 831	65 145
20	Jibannagar	3 14	9 120	4 082	81 253

MAP 1
MAP OF SOUTH-WESTERN BENGAL SHOWING THE INDIVIDUAL
CENTRES FOR THE ANALYSIS OF CHOLERA
MORTALITY FIGURES



The results shown in Table II are interesting. In almost all the centres the mean monthly and yearly variances are significantly greater than the respective residuals, when tested by means of Z-test. This shows that seasonal and yearly factors govern most of the variation in cholera incidence in these *thanas*.

In none of the *thanas* yearly variation or both yearly and monthly variations are insignificant. In one *thana* only, namely Kharagpur, monthly variance is insignificant.

Contiguous thanas in which, after separation of the monthly and yearly variances, the residuals are small and large respectively

Midnapur district		MEAN VARIANCE		
		Monthly	Yearly	Residual
(1)	Tamluk cum Moyna	224 500	84 284	7 548
	Maslandpur (Maisadal)	348 453	140 068	48 717
(2)	Pingla cum Sabang	45 804	23 895	6 969
	Pataspur	223 358	167 448	85 926

In the case of Maslandpur and Pataspur, separation of monthly and yearly variances from the total variance did not result in the reduction of the residual variance to the extent that it took place in their respective neighbouring *thanas*. Thus in case of both, further investigation seems to be necessary to explain why the residual variance should remain so high. The fact that, even between neighbouring *thanas*, figures for the residual variance show such differences seems to emphasize the necessity of re-casting the administrative units into areas of similar cholera experience before systematic study can be undertaken. This raises two questions, namely how many and which *thanas* may be included in each district without introducing definite heterogeneity and how to estimate variances for the combined areas. The method adopted for the solution of these problems is the trivariate analysis of variance. The procedure adopted in this analysis is summarized in Table III:—

TABLE III
Trivariate analysis of variance

Source of variation	Degrees of freedom	Sum of squares	Mean square	Contribution to total variance
1 Thanas	$n-1$	$S(x_w - \bar{x})^2$	$\frac{1}{n-1} \times S(x_w - \bar{x})^2$	$= P_1 \frac{P_1 - P_7}{12 \times 32}$
2 Months	11	$S(\bar{x}_u - \bar{x})^2$	$\frac{1}{11} \times S(\bar{x}_u - \bar{x})^2$	$= P_2 \frac{P_2 - P_7}{n \times 32}$
3 Years	31	$S(x_t - \bar{x})^2$	$\frac{1}{31} \times S(x_t - \bar{x})^2$	$= P_3 \frac{P_3 - P_7}{n \times 12}$
Interaction				
4 Thanas & years	$31(n-1)$	$S(x_{tw} - \bar{x}_w - \bar{x}_t + \bar{x})^2$	$\frac{1}{31(n-1)} S(\bar{x}_{tw} - \bar{x}_w - \bar{x}_t + \bar{x})^2$	$= P_4 \frac{P_4 - P_7}{12}$
5 Thanas & months	$11(n-1)$	$S(\bar{x}_{uw} - \bar{x}_w - \bar{x}_u + \bar{x})^2$	$\frac{1}{11(n-1)} S(\bar{x}_{uw} - \bar{x}_w - \bar{x}_u + \bar{x})^2$	$= P_5 \frac{P_5 - P_7}{32}$
6 Months & years	341	$S(\bar{x}_{ut} - \bar{x}_t - \bar{x}_u + \bar{x})^2$	$\frac{1}{341} S(\bar{x}_{ut} - \bar{x}_t - \bar{x}_u + \bar{x})^2$	$= P_6 \frac{P_6 - P_7}{n}$
7 Residual	$341(n-1)$	$S(x_{utw} - \bar{x}_{tw} - \bar{x}_w - \bar{x}_u + \bar{x}_{tu} + \bar{x}_{tw} + \bar{x}_{uw} - \bar{x})^2$	$\frac{1}{341(n-1)} S(x_{utw} - \bar{x}_{tw} - \bar{x}_w - \bar{x}_u + \bar{x}_{tu} + \bar{x}_{tw} + \bar{x}_{uw} - \bar{x})^2$	$= P_7$
TOTAL	$384(n-1)$	$S(x_{utw} - \bar{x})^2$		

S means summation over all the values x_{uvw} means the monthly value of a particular year and *thana*, \bar{x}_w means the mean when months and years are suppressed in any *thana*, \bar{x}_u the mean obtained by suppressing only years and *thanas*, \bar{x}_v the mean obtained by suppressing the months and *thanas*. In the same way \bar{x}_{uv} , \bar{x}_{vw} , \bar{x}_{uw} denote respectively the means obtained by suppressing *thana*, month and year. The contribution that each source of variation makes to the total variability is shown in the last column. We suppose in general that n *thanas* are grouped.

By this method the variation is split up into seven components described below and the significance of the first six may be tested against the residual by Z-test

SOURCE OF VARIATION

1 Between means of *thanas*—this gives an estimate of the difference between the various centres constituting the *thanas*

2 Between means of months—this is an estimate of the importance of seasonal factors over the whole group of *thanas*

3 Between means of years—this measures the epidemicity of the group as a whole

4 Interaction between *thanas* and months—this measures the extent of similarity between *thanas* as regards the seasonal incidence in each of them.

5 Interaction between *thanas* and years—this measures the extent of similarity of experience of various *thanas* with regard to their epidemicity

6 Interaction between months and years—this gives a measure of the similarity of mean seasonal curves from year to year considering the group as a whole

In order to form cholera districts a number of contiguous *thanas*, which on inspection appeared to be similar, were grouped together and the trivariate analysis was carried out. On testing a few groups, by this method it was found that Fisher's Z-test as a criterion for significance was too stringent for these data. A simpler procedure was therefore adopted which consisted of the calculation of the percentage contribution made by each source of variation to the total variability. The group was considered homogeneous if the percentage contributions of (1), (4) and (5) fell below 5 per cent of the total variance. The grouping was first carried out by a comparison of the characteristics of contiguous *thanas* by inspection and the group was subjected to trivariate analysis. In case the various estimates were too high to permit their combination certain *thanas* were excluded till by a process of trial and error the results obtained were satisfactory. The homogeneous districts as finally worked out are shown in Table IV and Map 2.

TABLE IV

Distribution of southern Bengal into homogeneous cholera districts

Number of the cholera district	Thanas comprising the cholera districts		Total number of thanas in the cholera district	Total population of the cholera district in 1931	Area in square miles of the cholera district
	District	Thanas			
1	Birbhum	Murari, Nalhati, Rampurhat, Mayureswar, Labipur, Suri group	8	1,329,504	2,000
	Burdwan	Asansol group, Raniganj			
2	Burdwan	Ketugram, Katwa, Mangalkote, Monteswar, Purbasthali	9	668,008	1,210
	Birbhum	Naucoor			
3	Nadia	Kaliganj, Tehatta, Melerpur	19	1,357,631	2,500
	Nadia	Nayapara, Damurhuda, Chuadanga, Gangauli, Krishanganj, Chapra group, Bantipur, Karimpur, Daulatpur, Alamdanga, Kumar khali, Kustha, Jibannagar, Ranaghat, Hauskhal, Chakdaha, Nakasipara			
4	Burdwan	Kalna	20	2,385,553	4,131
	Hooghly	Belgarhi			
	Burdwan	Kakesa, Augram, Galsi, Burdwan group, Khandagholi, Rana, Jamalpur			
	Bankura	Borjora group, Bishnupur group, Bankura group			
	Midnapur	Chandrakona, Keshpur			
Hooghly	Hooghly	Goghat, Arambagh, Pandua, Danaikhal group, Tarakeswar group, Serampur group, Krishnagar, Hooghly group			

Epidemiology of Cholera in Bengal

TABLE IV—*contd*

Number of the cholera district	Thanas comprising the cholera districts		Total number of thanas in the cholera district	Total population of the cholera district in 1931	Area in square miles of the cholera district
	District	Thanas			
5	Bankura	Onda group, Simlupal	7	612,061	1,714
	Midnapur	Garhbeta, Salbani, Gopiballavpur, Midnapur rural, Kharagpur			
6	24 Parganas	Nahati group, Habra, Deganga, Barasat, Barrackpore, Bhangar, Sonerpur	17	1,870,905	1,898
	Howrah	Jagatballavpur group, Domjur group, Amtoli group			
7	Hooghly	Khanakul	16	1,966,602	2,100
	Midnapur	Ghatal, Daspur, Panskura, Debra, Pingla group, Narayangarh group			
	24 Parganas	Barurpur group, Bishnupur, Falta, Diamond Harbour, Budge Budge			
	Howrah	Shampur group, Bagnan, Uluberia group			
8	Midnapur	Tamluk group, Maisadal, Sutahata, Bhagwanpur group, Nandigram, Egra group, Ramnagar, Pataspur	4	761,538	1,710
	24 Parganas	Magrahat, Canning (Matla), Jaynagar, Kulpi group			

9	Khulna	Kalaroa, Satkhura, Kaliganj Group	4	500,400	720
	24 Parganas	Basirhat			
10	Khulna	Magura, Dacopo group, Rampal, Bagerhat group, Damuria, Batiaghata, Mollahat	8	1,004,037	1,522
	Backerganj	Sarupkati group			
11	Backerganj	Nalchiti, Backerganj, Pirojpur, Mathbaria, Bhandaria, Patuakhali, Bauphal, Amtoli, Golachipa, Barisal, Melendiganj, Jhalakati	13	1,479,183	1,941
	Khulna	Morelganj group			
12	Bankura	Khatra group	1	257,340	675
13	Midnapur	Danton group	1	115,059	225
14	24 Parganas	Behula	1	44,008	20
15	24 Parganas	Sarupnagar group	1	130,034	164
16	24 Parganas	Huroa group	1	106,711	276
17	Khulna	Asasuni	1	95,612	158
18	Khulna	Khulna group	1	180,309	185
19	Backerganj	Bhola group	1	423,723	689

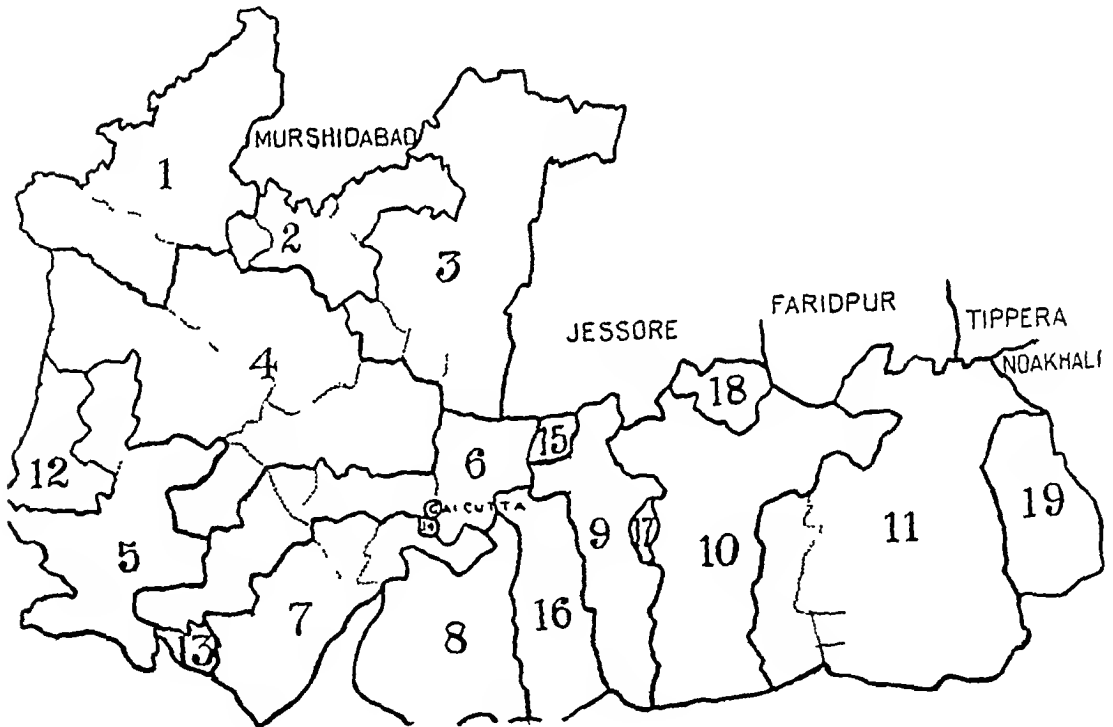
It will be observed that the size of the districts so formed is very variable and in certain areas a single *thana* constitutes a district. It is also obvious

MAP 2

MAP OF SOUTH-WEST BENGAL SHOWING AREAS OF
HOMOGENEOUS CHOLERA MORTALITY EXPERIENCE

(FOR THE PERIOD 1901-1932)

(Dotted lines indicate boundaries of the
administrative districts)



that there is no correspondence between the administrative and the cholera districts

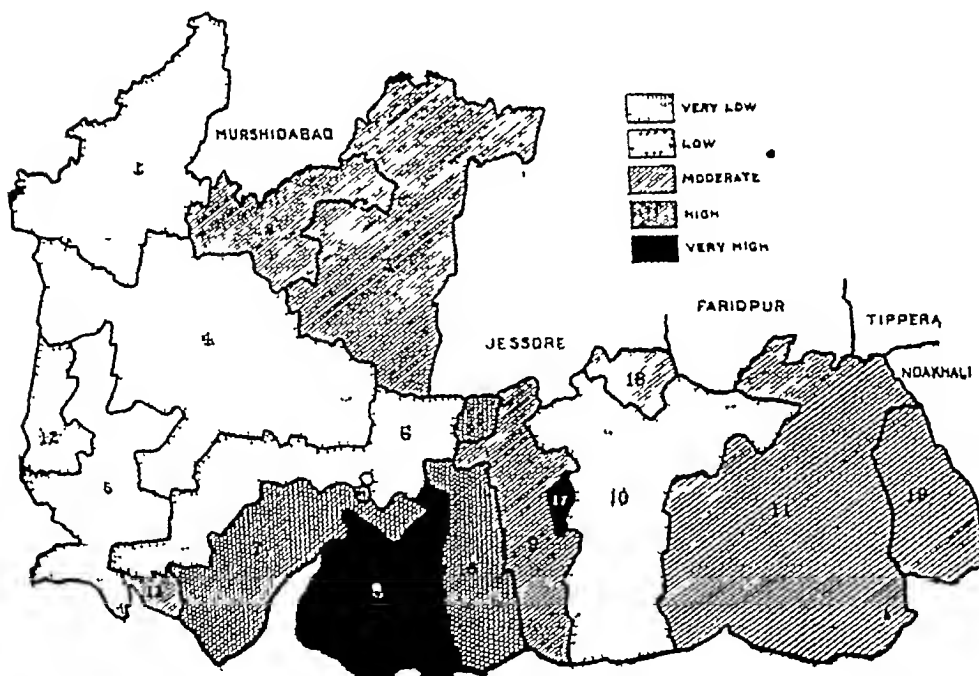
EPIDEMIOLOGICAL CHARACTERISTICS OF THE HOMOGENEOUS CHOLERA DISTRICTS

I Mean incidence (*vide* Map 3)

It will be seen that with the exception of district No 10 which shows low incidence all the deltaic districts show moderate to high incidence, while the western districts have a lower cholera mortality

MAP 3

MAP OF SOUTH-WEST BENGAL SHOWING THE MEAN INCIDENCE OF THE HOMOGENEOUS CHOLERA DISTRICTS



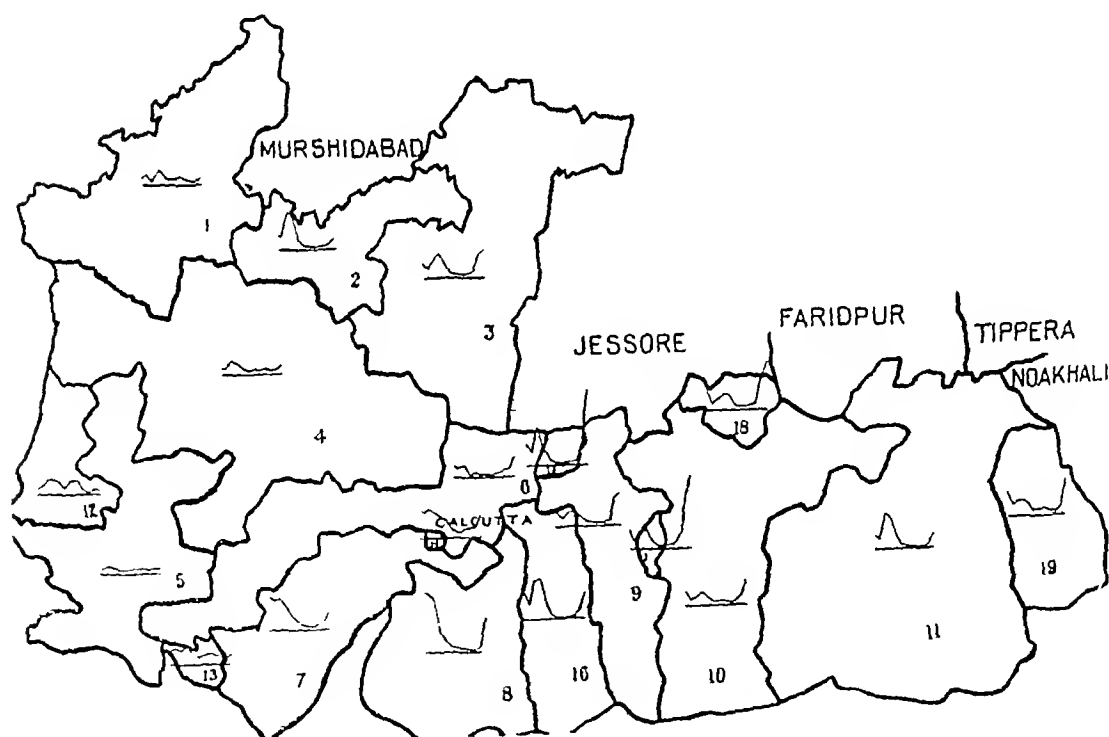
II *Seasonal curves (vide Map 4)*

The general description of the various types of the curves has already been given in a previous communication (Lal *et al*, *loc cit*)

MAP 4

MAP OF SOUTH-WEST BENGAL SHOWING SEASONAL
CURVES FOR HOMOGENEOUS CHOLERA DISTRICTS

(FOR THE PERIOD 1901-1932)



SUMMARY

1 Taking *thana* as a unit the total variability of cholera incidence has been split up into three variables, viz seasonal, yearly and residual, by the method of analysis of variance

2 Contiguous *thanas* showing similarity in respect of different types of variation, mean cholera incidence and type of seasonal curve have been combined and the districts so obtained tested for homogeneity by trivariate analysis of variance Those satisfying tests of homogeneity have been constituted into cholera districts

3 The sizes of the homogeneous cholera districts vary from individual *thanas* to a combination of 20 centres The boundaries of the cholera districts and the administrative districts do not correspond

4 The epidemiological characteristics of the homogeneous cholera districts have been generally discussed

5 It is believed that the re-distribution of the area into homogeneous cholera districts gives a basis for investigation of the underlying factors in the natural history of cholera which are responsible for the observed variations

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STATISTICAL INQUIRY INTO THE EPIDEMIOLOGY OF CHOLERA IN BENGAL

Part III

ENDEMICITY AND EPIDEMICITY OF THE HOMOGENEOUS CHOLERA DISTRICTS

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IN the previous communication (Lal *et al*, 1941b) the total variability of cholera mortality in a locality has been split up into three components, viz (1) monthly, (2) yearly and (3) residual variances, and the results so obtained have been utilized in the formation of homogeneous cholera districts and the description of other epidemiological characteristics. Here we propose to utilize, for the purpose of studying the epidemic and endemic nature of these homogeneous cholera districts, the results obtained by the method of analysis of variance.

An area can be said to be endemic for cholera when the disease is constantly present and it is said to be non-endemic when long intervals of time separate the

occurrence of outbreaks. An endemic area may be subject to epidemic visitations, the intervals showing relatively low incidence of cholera. The essential feature of endemicity is continuity in time of disease prevalence though, from the practical point of view, the intervals between outbreaks never tend to zero. 'Endemic' and 'non-endemic' are relative terms, the interval in the case of the former being sufficiently short to permit of the assumption of persistence of infection in the community and, in the latter, the interval being of such duration as to suggest that infection died out. In regard to endemicity, therefore, we have to take into consideration intervals of absence of cholera both within and between the years during the period under investigation. We have to examine the distribution of cholera figures against time as a variable, in order to determine whether infection has been persistent or not. Discontinuity of infection can be judged only from the absence of cholera mortality figures and certain arbitrary standards have to be prescribed.

We cannot, however, strictly apply the above criterion of endemicity to the homogeneous districts. As each district is composed of several *thanas*, it is highly improbable that there will be absence of cholera mortality simultaneously in all the *thanas* over a number of consecutive months. As a matter of fact, in the case of homogeneous districts with more than one *thana* absence of cholera mortality even for two consecutive months is very rare. Since all the cholera deaths occurring in the constituent *thanas* of a district are added up, the fact that a single *thana* registered cholera deaths, while the others were entirely free from cholera mortality, would mean that in our analysis we will have to assume the persistence of infection for that particular month over the district as a whole. Such a procedure emphasizes the random behaviour of one or two *thanas* in a district, while it completely ignores the behaviour of the majority of the *thanas* in the district. In order to get over this difficulty the following procedure has been adopted —

For each *thana* a frequency distribution was prepared showing the occurrence of varying periods of absence of cholera deaths. These distributions are presented in *Appendix I*. The frequencies corresponding to each type of interval were added for all the *thanas* constituting each homogeneous cholera district. It is likely that the total frequency for any particular interval may be correlated with two factors, namely, the total population of a homogeneous district and the total number of *thanas* included in the district. In order to allow for the effect of these two factors, the data for the 19 homogeneous districts have been used to work out a multiple regression equation for each type of interval. Table I gives the partial regressions together with their respective standard errors and the corresponding values of *t*, beginning from a period of one month to a period of 14 months during which there were no cholera deaths. As intervals above 14 months are very rare the analysis was not carried out beyond this interval.

The number of degrees of freedom available for the calculation of the standard error in each case is 16 because the number of districts is 19. The expected 5 per cent value of *t* for 16 degrees of freedom is 2.120.

TABLE I

Regression of frequencies of intervals for which cholera was totally absent on population and number of thanas, standard error of regression and value of t

Interval of months.	POPULATION			NUMBER OF thanas		
	Regression b_1	Standard error	t	Regression b_2	Standard error	t
1	+ 0 00005544	0 0000229	2 422	+ 14 6729272	2 4297	6 039
2	-0 00002050	0 0000170	1 204	+ 11 0622626	1 8073	6 129
3	-0 00002493	0 0000157	1 587	+ 7 9368171	1 6674	4 760
4	-0 00005227	0 0000134	3 915	+ 8 7533745	1 4170	6 178
5	-0 00003047	0 0000134	3 758	+ 8 0105534	1 4268	5 624
6	-0 00003242	0 0000071	4 586	+ 4 9400756	0 7490	6 588
7	-0 00001495	0 0000046	3 222	+ 2 2223347	0 4929	4 309
8	-0 00001916	0 0000040	4 826	+ 2 6782567	0 3973	6 741
9	-0 00001193	0 0000033	3 571	+ 1 7904637	0 3646	4 935
10	-0 00001277	0 0000019	6 650	+ 1 5722984	0 2036	7 723
11	-0 00000392	0 0000015	2 578	+ 0 4896897	0 1619	3 025
12	-0 00000281	0 0000084	3 341	+ 0 4380674	0 0888	4 933
13	-0 00000537	0 00000083	6 466	+ 0 1414486	0 0882	1 604
14	-0 00000693	0 00000069	10 0390	+ 0 1201985	0 0730	1 647

It will be noticed from Table I that the size of the population has no significant effect on the frequency of intervals of two months and three months. On the other hand the number of thanas constituting a homogeneous district appears to have no significant effect on the frequency of intervals of 13 and 14 months. In these cases better result is expected by dropping the independent variable corresponding to which the regression is not significant and adjusting the regression for the other variable. The adjusted regressions are given in Table II.

TABLE II

Adjusted regressions

Interval in months	Population b_1	Interval in months	Number of <i>thanas</i> b_2
13	+ 0 000000716	2	+ 9 016665
14	+ 0 000000372	3	+ 5 448906

We can now calculate the expected frequencies of intervals of one, two, up to fourteen months for each homogeneous district from the following regression equation —

$$y = (\bar{y} - b_1 \bar{x}_1 - b_2 \bar{x}_2) + b_1 x_1 + b_2 x_2$$

where y = expected frequency

\bar{y} = mean of observed frequencies for 19 homogeneous districts

\bar{x}_1 = mean of population of 19 homogeneous districts

\bar{x}_2 = mean of number of *thanas* in 19 homogeneous districts

x_1 = population of a homogeneous district

x_2 = number of *thanas* in a homogeneous district

In the case of intervals where frequencies of occurrence are not affected by either of the variables, population and number of *thanas*, we have to modify the above equation by leaving out that particular variable and using the adjusted values for the regression due to the other variable. From these regression equations the expected values of the frequencies of different intervals were calculated district by district. As it is impossible to have negative frequencies, these were treated as zero and the difference between the expected and the observed values (expected-observed) is given in *Appendix II*. The positive or negative values of these differences will show whether the frequency of a particular interval in a district is in excess or in defect of what is likely to occur normally, after due allowance has been made for the size of the population and the number of *thanas* of the district. This leads us to the following criterion of judging whether a homogeneous district is endemic or non-endemic.

A homogeneous district is to be classed as endemic or non-endemic according as the mean of the differences for all the intervals is negative or positive. A break of a single month, however, may be due to pure chance alone, and therefore the difference corresponding to the interval of one month has been left out in calculating the mean difference.

Table III gives the mean difference for each homogeneous district and the classification of the homogeneous districts into endemic and non-endemic areas according to the above criterion (*vide* Map).

MAP
 OF SOUTH-WEST BENGAL SHOWING ENDEMICITY AND
 EPIDEMICITY OF THE HOMOGENEOUS
 CHOLERA DISTRICTS

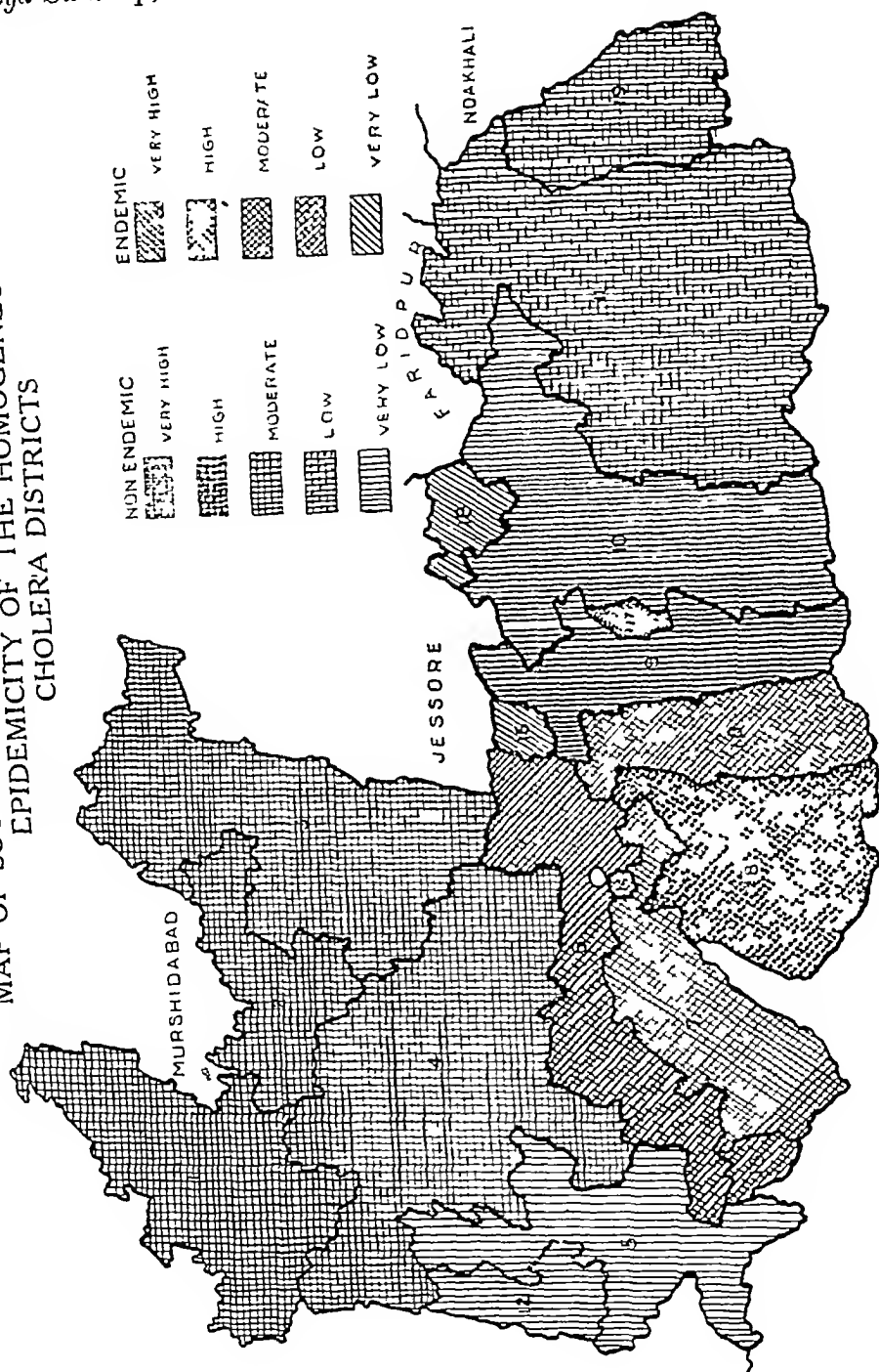


TABLE III

Mean difference and endemicity of homogeneous districts

Homogeneous district	Mean difference	Endemicity
1	+ 8 6955	Non endemic
2	+ 3 1749	do
3	+ 7 8239	do
4	+ 5 5871	do
5	+ 2 4324	do
6	-8 0834	Endemic
7	-15 2258	do
8	-1 1419	do
9	+ 1 0438	Non endemic
10	+ 0 7332	do
11	+ 2 4183	do
12	+ 0 9989	do
13	-1 9824	Endemic
14	-3 2453	do
15	-1 2889	do
16	-0 8839	do
17	-1 5237	do
18	-1 6040	do
19	+ 0 0084	Non endemic

Out of the 19 homogeneous districts nine are endemic. Of these, again, two districts, namely Nos 17 and 18, are isolated *thanas*, while the seven other endemic districts form one continuous tract of land near the Bay of Bengal.

In the foregoing method of classification of homogeneous cholera districts into endemic and non-endemic areas we have eliminated the effect of population and number of *thanas* in a district. It may be considered more logical to eliminate the effect of the area of a district rather than to the number of *thanas* included in the

district In order to investigate this point partial regressions have been worked out with area and population as the independent variables From the values of t given in Table IV, we can judge the significance of the partial regression

TABLE IV

Values of t for testing the significance of partial regression due to population and area

Interval of months	Area	VALUES OF t
		Population
1	2 221*	3 708*
2	3 703*	0 404
3	2 483*	0 771
4	1 897	2 091
5	1 810	0 573
6	1 857	4 677
7	2 737*	1 602
8	1 721	0 823
9	3 147*	1 850
10	2 149*	1 011
11	2 538*	2 051
12	0 415	0 533
13	4 078*	2 478*
14	3 977*	2 874*

* Note —Value of t marked with an asterisk is significant at the 5 per cent level

A comparison of Tables I and V would show that on the whole a better result would be obtained if the regression equation contains the number of *thanas* and not the area

A third method of approach towards the same problem would be to treat each homogeneous district separately and eliminate the effect of population and area of the *thanas* in a district on the frequency of the different intervals of absence of cholera mortality

Such a procedure would necessarily require that each district should consist of a number of *thanas* This is, however, not the case There are a number of

homogeneous districts each of which consists of one *thana* only. We have, nevertheless, worked out partial regressions for each homogeneous district having sufficient number of *thanas*, with population and area as the independent variables. The *t* test applied to these regressions has failed to establish their significance in all the cases. Thus, we may conclude that with the available data the first method of classification will perhaps give the best result.

Next, the problem was to provide for objective measures for the further subdivision of the endemic and non-endemic areas on the basis of their varying chances of epidemic outbreaks. The frequency of epidemic outbreaks is likely to vary from area to area both in the case of the endemic and of the non-endemic areas. For formulating a scheme of classification we are concerned with the yearly variance values and not the monthly and residual variances. The monthly variance gives an average measure of the variability of individual monthly values round each yearly mean without reference to the distribution of these values in relation to time. Hence the monthly variance does not give, by itself, a measure of endemicity or of continuity of infection. The monthly variance was not therefore considered in our earlier discussion regarding endemic and non-endemic areas. On the other hand, the yearly variance has a definite bearing on the problem of epidemicity in view of the fact that the mean yearly incidence is bound to vary widely from year to year when a locality is subject to epidemics. The proposed subdivision of endemic and non-endemic areas, on the basis of smaller and greater chances of epidemic visitations, has therefore to be carried out on the values of the yearly variance.

In connection with formulation of suitable methods of forecast the periodicity of cholera mortality was investigated by periodogram analysis. In no case was any periodicity demonstrated. Epidemic outbreaks may be considered to be deviations from the normal cholera experience of the locality under investigation, whether it belongs to the endemic or non-endemic group. Failure to demonstrate periodicity in cholera prevalence may therefore be interpreted as a suggestion that the outbreaks of epidemic are random occurrences. If this be so, the application of levels of probability based on the standard error of the mean yearly variances may be made the method of subdivisions of the endemic and non-endemic groups.

Before we can set up these levels we have to see if the yearly variance of a homogeneous district is influenced by the mean incidence, population and number of *thanas* of the district. Three correlations were worked out between yearly variance and each one of the other three factors. The values of the correlations are —

Correlation between yearly variance and	Coefficient of correlation
Mean incidence	0.0306
Population	0.0007
Number of <i>thanas</i>	0.0504

None of the coefficients is significant

Thus, on the evidence we have before us, we shall be justified in assuming that the outbreaks of epidemics are random occurrences

The mean of the 19 yearly variances is 484.17 and their standard error is 611.89. With such large value of the standard error we have taken eighth part of it as the basis for our levels. Writing $m = 484.17$ and $s = 76.49$ (one-eighth of 611.89) we have chosen the following levels of epidemicity for both the endemic and the non-endemic areas —

Epidemicity	Range of yearly variance	Value of range
Very high	$m + 3s$ and higher	713.64 and higher
High	$m + s$ to $m + 3s$	560.66 to 713.64
Moderate	$m + s$ to $m - s$	407.68 to 560.66
Low	$m - 3s$ to $m - s$	254.70 to 407.68
Very low	$m - 3s$ and lower	254.70 and lower

A classification of the endemic and non-endemic areas on these lines is given in Table V —

TABLE V

Classification of homogeneous districts into endemic and non-endemic areas with varying degrees of epidemicity

Homogeneous district number	Endemicity	Degree of epidemicity
1	Non endemic	High
2	"	Very high.
3	"	" "
4	"	Low
5	"	Very low
6	Endemic	Low
7	"	Very high
8	"	" "
9	Non endemic	Very low

TABLE V—*concl'd*

Homogeneous district number	Endemicity	Degree of epidemicity
10	Non endemic	Very low
11	"	Moderate
12	"	Very low
13	Endemic	Very high
14	"	Very low
15	"	" "
16	"	" "
17	"	" "
18	"	" "
19	Non endemic	" "

SUMMARY

The endemicity and epidemicity of the cholera homogeneous districts have been investigated. A frequency distribution of varying intervals of absence of cholera has been worked out for each *thana* individually and for the *thanas* constituting each homogeneous district collectively. Since the gross endemicity so determined is subject to variations due to differences in population, area and the number of *thanas* constituting the districts the effects of these factors have been eliminated by the method of partial regression. The net endemicity so determined has been used to classify the homogeneous districts into endemic and non-endemic. Using the yearly variance as a measure of epidemicity the homogeneous districts have been divided into five arbitrary groups. Thus, the epidemic and endemic characters of various homogeneous cholera districts of south-west Bengal have been defined.

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Total number of zeroes occurring in succession in the centres constituting each homogeneous district and the population and total number of centres in the district

[illegible]

APPENDIX I—concl

[illegible]

APPENDIX II

*Statement showing the deviations of monthly and yearly variances
of the observed respective values (Correction for varying
values of mean incidence in each centre)*

Number	District and centre	Monthly variance (observed expected)	Yearly variance (observed expected)
<i>Birbhum</i>			
1	Mayureswar	-0 668	+7 748
2	Nanoor	+2 970	+3 776
3	Rampurhat	-0 692	+7 934
4	Nalhati	-3 054	+3 341
5	Suri group	-0 566	+6 158
6	Labpur	-1 732	+6 120
7	Muraroi	-1 890	+1 667
<i>Burdwan</i>			
1	Khandaghosh	-0 063	-0 154
2	Ausgram	+0 232	+0 902
3	Burdwan group	+0 924	-0 785
4	Galsi	-0 205	+0 320
5	Kaksa	+1 784	+0 796
6	Monteswar	+4 085	+9 800
7	Katwa	+3 004	+4 121
8	Purbasthali	-0 935	+1 874
9	Mangalkote	+6 918	+4 416
10	Ketugram	+4 460	+0 534
11	Kalna	-1 291	-0 850
12	Asansol group	+0 868	+3 496
13	Raniganj group	+0 467	+4 026
14	Jamalpur	+1 120	-0 647
15	Raina	-0 157	-0 523

APPENDIX II—contd

Number	District and centre	Monthly variance (observed- expected)	Yearly variance (observed- expected)
<i>Bankura</i>			
1	Simlapal	+3 517	—0 033
2	Onda, Taldanga	+3 384	+0 160
3	Barjora group	+0 011	+1 901
4	Khatra group	—3 207	+16 756
5	Bankura, Chatna	+0 696	+0 583
6	Vishnupur group	+1 670	—0 329
<i>Midnapore</i>			
1	Maisadal	—10 390	—1 002
2	Egra, Contai	—2 643	—1 944
3	Narayangarh group	—0 602	—0 504
4	Nandigram	—10 880	+0 203
5	Tamluk, Moyna	—8 593	—0 729
6	Ramnagar	—4 286	+0 410
7	Bhagwanpur group	—6 782	—0 489
8	Midnapore	+2 773	+0 006
9	Debra	—2 325	—1 560
10	Keshpur	—1 764	—0 751
11	Salbani	+6 138	+0 593
12	Garbetta	+4 482	+0 128
13	Daspur	—3 258	—1 538
14	Danton group	—7 888	+1 258
15	Pingla group	—4 654	—2 368
16	Kharagpur	+4 047	+0 138
17	Chandrakona	+1 656	—1 083
18	Ghatal	—2 886	+0 210

APPENDIX II—contd

Number	District and centre	Monthly variance (observed expected)	Yearly variance (observed expected)
<i>Midnapore—concl</i>			
19	Panskura	-7 134	-1 808
20	Gopiballarpur	+5 929	+0 601
21	Sutabhatta	-12 071	-2 576
22	Patashpur	-12 779	-4 044
<i>24 Parganas</i>			
1	Falta	-4 402	-2 400
2	Diamond Harbour	-0 245	-2 521
3	Deganga	+1 323	-1 612
4	Magrahat	+11 390	+1 739
5	Bishnupur	-3 627	-3 704
6	Bhangar	-4 426	-2 648
7	Haroa group	-3 120	-0 027
8	Habra	+0 920	-2 302
9	Sonarpur	-1 323	-2 975
10	Sarupnagar group	+10 375	-2 737
11	Baruipur group	+0 933	-3 757
12	Basirhat	+3 543	-3 593
13	Naihati group	-0 365	-2 207
14	Matla	-9 385	+3 237
15	Kulpi	+14 995	-0 306
16	Behala	-15 597	-2 499
17	Barasat	-1 535	-2 797
18	Barrackpore group	-2 642	-0 151
19	Jamagar	+15 026	+6 007

APPENDIX II—contd

Number	District and centre	Monthly variance (observed expected)	Yearly variance (observed- expected)
<i>Khulna</i>			
1	Baighata	-1 480	-2 365
2	Damuria	-5 566	-2 317
3	Paikacha group	-3 437	-1 811
4	Magura	-4 315	-2 660
5	Satkhira	+5 809	-1 364
6	Kalaroa	+1 283	-1 242
7	Bagerhat group	-2 307	-2 452
8	Khulna group	+0 279	-3 861
9	Mollahat	+1 332	-1 055
10	Morelganj	+3 414	-0 694
11	Kaliganj group	-1 512	-1 380
12	Asasuni	+10 060	+1 804
13	Ramphal	-1 027	-1 544
<i>Hooghly</i>			
1	Arambagh group	-1 227	-0 576
2	Krishnanagar group	+2 676	-0 792
3	Dadpur	-0 079	-1 182
4	Serampur	+7 350	-1 155
5	Hooghly, Magra	-0 849	-1 964
6	Tarakeswar group	-0 303	-1 496
7	Pandua	+0 648	-1 539
8	Khanakul	-3 319	-0 932
9	Balgarh	-0 716	-1 633
10	Goghat group	+1 265	-0 387

APPENDIX II—contd

Number	District and centre	Monthly variance (observed expected)	Yearly variance (observed expected)
<i>Howrah</i>			
1	Shampur	-7 833	-2 478
2	Uluberia group	-8 122	-3 413
3	Amta group	-5 908	-2 376
4	Dumjar group	-7 423	-3 233
5	Jagatballavpur group	-6 837	-2 835
6	Bagnan	-9 679	-3 750
<i>Backerganj</i>			
1	Nalchuti	+0 833	-2 450
2	Backerganj	+1 681	-1 736
3	Pirojpur	+3 992	+2 403
4	Sarupkati	-1 046	-1 145
5	Mathbaria	+0 492	+3 854
6	Bhandaria	+1 840	+1 064
7	Patuakhali	+7 737	-1 997
8	Bauphal	-1 672	-2 633
9	Amtoli	+7 348	-0 366
10	Galachipa	+3 691	-0 347
11	Barisal	+2 475	-2 531
12	Mehndiganj	-0 456	-2 694
13	Jhalakati	+1 610	-1 439
14	Bhola	-4 901	-0 447
<i>Nadia</i>			
1	Nayapara	-0 254	+0 436
2	Damurhuda	-2 430	+1 932

APPENDIX II—concl'd

Number	District and centre	Monthly variance (observed expected)	Yearly variance (observed expected)
<i>Nadia—concl'd</i>			
3	Chuadanga	−3 559	−0 106
4	Gangani	+0 491	+0 781
5	Kissengunj	+4 079	+0 354
6	Chapra group	+0 559	−1 385
7	Santipur	+0 902	−2 122
8	Naksipara	+0 682	+0 087
9	Tehatta	+0 964	+3 453
10	Kaliganj	+1 081	+2 098
11	Ranaghat	−0 066	−1 924
12	Karimpur	+1 337	+2 191
13	Daulatpur	+0 811	+3 674
14	Alamdanga	+0 087	−0 840
15	Kumarkhali	+1 328	−0 848
16	Meherpur	−4 308	+2 089
17	Chakdaha	+0 593	+3 443
18	Kusthia	−0 087	−2 736
19	Hanskhali	+2 469	−0 714
20	Jibannagar	−0 963	−1 200

HÆMOLYTIC *STREPTOCOCCI* IN THE THROAT OF APPARENTLY HEALTHY PERSONS ITS INCIDENCE AND CAUSAL RELATIONSHIP TO PUERPERAL INFECTION

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WITHIN recent years the importance of hæmolytic *streptococci* in puerperal infection has been generally recognized. Of all the different possible sources of infection investigated by numerous authors, viz Smith (1931, 1933), Courmont and Sédallion (1931), Paine (1931, 1935), Colebrook, D (1935), Hare (1935), Hare and Maxted (1935), Colebrook, L, Maxted and Johns (1935), Cruickshank (1935), the human respiratory passages seem to be the only important reservoir of those strains of hæmolytic *streptococci* capable of causing severe puerperal infection. The vast majority of them are due to the transfer of the organisms from the throats of contacts during or shortly after labour, directly by droplet infection or indirectly through the use of soiled handkerchief, hands, basin, towel, etc. Apart from the fact that the attendants suffering from apparent 'septic throat', it is likely that they may be 'healthy carriers', i.e. persons whose pharynx harbour hæmolytic *streptococci* but show no sign of infection. These organisms in healthy carriers may give rise to a mild, severe, or even fatal infection when the environment is favourable. The mere presence of hæmolytic *streptococci* in the throat is not of any serious import unless it is shown that they possess characters similar to those strains recovered from definite human infections.

Many workers have described the presence of hæmolytic *streptococci* in the throat of normal persons in different countries. The percentage of healthy carriers

in a normal population varies between 47.7 (Paine, 1935) and 7.7 (Blackburn *et al.*, 1930, quoted by Paine, 1935). So far in India the only observation has been recorded by Pasricha and Panja (1940). With the exception of few no endeavour has been made to determine the potential human pathogens.

Of the various methods which have been devised from time to time for the differentiation of hæmolytic *streptococci* of potential pathogenicity to man from non-pathogenic strains or strains of animal origin, the precipitin test of Lancefield is now regarded as the most valuable.

The present work records a biochemical and serological study of hæmolytic *streptococci* isolated from the throat of apparently healthy persons to determine what proportion of them possess characters similar to those strains of definite human infections.

SOURCE OF STRAINS

Samples were taken from persons who had no apparent infection of the throat or any recent history of the same. They consisted of medical students, hospital and laboratory staff, handy women, patients confined in the hospital, hospital out-patients and nurses in the hospital.

TECHNIQUE

Swabs after moistening them with 0.85 per cent saline were rubbed over the tonsils and the posterior pharyngeal wall. The importance of securing a really effective contact between the tonsil and the swab cannot be over-estimated. It is for this reason that the swabs were taken in favourable circumstances by one accustomed to the technique as stressed by Colebrook, D (*loc cit.*). In summer the tubes containing the swabs were immediately kept in a thermos flask containing ice before bringing it to the Laboratory. The swabs were inoculated within an hour in 5 per cent horse blood-agar plates and incubated anaerobically in McIntosh and Fildes' jar for 18 to 20 hours at 37°C. In the beginning, duplicate platings were made and incubated both aerobically and anaerobically. The anaerobic cultures gave a higher percentage of positive results and in no case was a positive result obtained under aerobic condition which was not at the same time found positive under anaerobic condition. So, latterly only one plate was used and incubated anaerobically. A further advantage of primary anaerobic culture is that the hæmolytic zones are very well defined and the growth of most of the other organisms is inhibited so that their distinction and isolation are much easier.

It is also particularly important to use horse blood as a standard technique for the purpose of identification and classification of hæmolytic *streptococci* (Brown, 1919). Likely colonies were transferred to 5 per cent serum Hartley broth and morphology studied, and replated to be sure of their purity. In some cases it was found necessary to replate when it was difficult to pick up isolated colonies, and subsequently the previous procedure was followed. The cultures were stocked in Robertson's cooked meat media at 5°C.

TABLE I

Ninety-two strains isolated from 480 swabs

Number of strains	PRECIPITATION WITH SERA OF GROUP										Formation of soluble haemolysin	Final pH in 1 per cent dextrose broth	Reduction of methylene blue in milk	GROWTH IN		FERMENTATION OF					
	OF GROUP													10 per cent bile agar	40 per cent bile agar	Glucose	Lactose	Salicin	Mannitol	Trehalose	Sorbitol
	A	B	C	D	E	F	F	G	H	K											
41	+	-	-	*	*	-	-	-	-	-	+++	4.9-5.1	-	+	-	+	+	+	+	+	+
10	+	-	-	*	*	-	-	-	-	-	++	4.6-5.1	-	+	-	+	+	+	+	+	+
5	-	+	-	*	*	-	-	-	-	-	+ to ++	4.4-4.7	-	+	+	+	+	+	+	+	+
2	-	-	+	*	*	-	-	-	-	-	++	4.9-5.0	-	+	+	+	+	+	+	+	+
5	-	-	-	*	*	+	-	-	-	-	++	4.9-5.1	-	+	-	+	+	+	+	+	+
1	-	-	-	*	*	+	-	-	-	-	+	4.0	-	+	-	+	+	+	+	+	+
2	-	-	-	*	*	-	-	-	-	-	++	4.8-4.9	-	+	-	+	+	+	+	+	+
2	-	-	±	*	*	-	-	-	-	-	++	4.0-5.0	-	+	+	+	+	+	+	+	+
11	-	-	-	*	*	-	-	-	-	-	++	4.8-5.1	-	+	+	+	+	+	+	+	+
1	-	-	-	*	*	-	-	-	-	-	-	4.9	-	+	-	+	+	+	+	+	+
6	-	-	-	*	*	-	-	+	-	-	-	4.9-5.1	-	+	-	+	+	+	+	+	+
3	-	-	-	*	*	-	-	-	-	+	-	5.0-5.2	-	+	-	+	+	+	+	+	+
1	±	-	±	-	-	-	-	-	-	-	++	4.9	-	+	-	+	+	+	+	+	+
2	-	-	-	-	-	-	-	-	-	-	++ and	4.8-5.0	-	+	-	+	+	+	+	+	+
TOTALS 92	51	5	2	-	-	6	15	7	3												

* Not tested

± Faint reaction

The last three strains could not be classified.

EXPERIMENTAL PROCEDURES

Preparation of precipitin sera—The method described by Lancefield (1933, 1938) was followed. The antisera were tested against homologous or heterologous strains (Lancefield, *personal communication*) and control tests were done with each batch of antisera with the group sera kindly supplied by Dr. Lancefield.

Preparation of extract—The methods of Lancefield (*loc. cit.*) and Plummer (1935) were followed.

Precipitin test—In performing this test the more recent micro-technique of Lancefield (1938) with slight modification was adopted. Small clear-glass tubes with an internal diameter of about 3 mm. were used for the test. D and E sera were not used as a routine as groups of these organisms are not present in throat. Only in three strains where no definite conclusions could be arrived at, we used D and E sera.

Final pH in 1 per cent dextrose broth—The method of Avery and Cullen (1919) was followed. Readings were made at the end of 4 days' incubation using methyl red as indicator.

Reduction of methylene blue in milk—The method of Avery (1929) was used. Only one concentration of 1 in 5,000 methylene blue in milk was used. Readings were taken on the following day and at intervals up to one week.

Hæmolysin test—Equal volumes of culture (18 hours in 20 per cent serum Hartley broth) and 5 per cent washed horse red cells were put in the incubator at 37°C. for 2 hours and kept in the ice-box overnight. Positive result was indicated by complete hæmolysis.

Fermentation of sugars—One per cent of each of the following sugars was added to Hiss serum water with Andrade's indicator: lactose, glucose, mannitol, salicin, sorbitol and trehalose. 0.1 c.c. of 18-hour broth culture was added to the media and readings taken on the following day and at intervals up to one week. All anomalous readings were repeated. When no fermentation occurred care was taken to ascertain that growth had occurred.

Growth on blood agar containing bile—The method described by Lancefield (1933) was followed.

RESULT

The specific grouping presented in Table I were made entirely on the basis of the precipitin test. Table I shows that out of 92 strains isolated from 480 swabs 51 fell in group A, 5 in group B, 2 in group C, 6 in group F, 15 in group G, 7 in group H and 3 in group K. They were all specific. Two strains of group G gave a faint cross reaction with group C antisera, one strain gave a precipitate with four different antisera and the other two gave variable results on repeated occasions. The last three strains have been kept unclassified.

Table II gives the distribution of different groups of *streptococci* in the throat as obtained by other workers.

TABLE II

Author	Number of swabs	Number of strains	GROUPS										Unclassified
			A	B	C	D	E	F	G	H	K		
Hare (1935)		150	03	5	15			12	13	25	8	9	
Davis and Guzdar (1936)	788	†68+10	28		23				27	*	*		
Pasricha and Panja (1940)	300	54	33	*	*	*	*	*	*	*	*		
Present series	480	92	51	5	2			6	15	7	3	3	

* Not tested

† Sixty eight strains from 788 swabs and 10 from other series of throat swabs

The appearance on the surface of blood-agar plates in secondary cultures gave a rough indication of their identity

In group A—the colonies were pearly white, convex, circular with an entire edge or greyish and flat—with a zone of complete hæmolytic from 1 mm to 2 mm in diameter in 24 hours, with sharply-defined margins

In group B—the colonies were larger with a zone of hæmolytic in which a few RBC could be seen After keeping in the refrigerator overnight there was increase of the zone of hæmolytic which tended to be more complete Further another faint band of concentric hæmolytic developed around the complete zone of hæmolytic This is very distinctive

Groups C and G were roughly similar to group A but the margins were more diffuse and the zones of hæmolytic were wider

Groups F, H, K—the zones of hæmolytic were small and diffuse

In the *hæmolytic test*, groups A, C and G gave a complete hæmolytic, but the group B strains did not produce hæmolytic to that extent Groups F, H and K gave a very faint or no hæmolytic at all Group D or E strains were not encountered

Final pH in 1 per cent dextrose broth—This test was useful only in differentiating group B strains from others which gave a pH which could be easily distinguished from others

Reduction of methylene blue in milk—We have not been able to gain any information by this test

Growth on ox bile—This was useful in identifying group B organisms most of which grew in 40 per cent bile blood agar and others did not

Fermentation of sugars—The results are in accordance with Lancefield and Hare (1935) and Hare (*loc cit*). Our C strains gave reactions of human strains. One group G strain failed to ferment lactose and two strains of this group did not act on salicin.

DISCUSSION

From these results it will be observed that about 10 per cent of the apparently healthy persons have group A hæmolytic *streptococci* in the throat in this part of the country. Whether all these strains given the suitable environment would cause infection is difficult to say but it is definite that severe hæmolytic *streptococcal* infections are always due to group A.

Reference to recent publications on puerperal pyrexia shows that pathogenic hæmolytic *streptococci* are absent from the genital tract before labour and their presence after labour is attributable to its introduction from without. Of the numerous authors referred to above, Colebrook, D (*loc cit*) in the epidemiological study of *streptococcal* infection in puerperium has brought forward convincing evidence that the vast majority of such infections could be traced to contacts during labour with attendants harbouring these organisms in their throats. Then it is to be expected that the incidence of the several groups of *streptococci* from parturient women will show the same proportion of group distribution as found in the throat. This is of course influenced by other factors.

In the succeeding paper, these results are compared which indirectly corroborate the findings of the other authors that the source of puerperal hæmolytic *streptococcal* infection is primarily in the throat of human beings.

SUMMARY

1. Ninety-two strains of hæmolytic *streptococci* have been isolated from 480 apparently healthy persons.

2. The carrier rate of group A *streptococci* is approximately 10 per cent, and that of group G is 3.1 per cent—the incidence of other groups of *streptococci* has been determined.

3. The biochemical reactions of these strains have been described.

4. The causal relationship of *streptococci* to puerperal infection has been discussed.

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SIGNIFICANCE OF HÆMOLYTIC *STREPTOCOCCI* IN PARTURIENT WOMEN

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IN the preceding communication (Chatterjee and Mitter, 1941) the presence of hæmolytic *streptococci* of different groups in the throat of normal persons in this country has been noted and its relationship to puerperal infection discussed. In a review of the work by other investigations it was concluded that the human nasopharynx is the main reservoir of the pathogenic *streptococci* in nature and that they are not normally present in the vagina.

The normal vagina has a characteristic flora during the reproductive period consisting largely of lactobacilli of Döderlein type. The normal acid secretion of the vagina has a marked bactericidal action on many species of bacteria. Menge (Topley and Wilson, 1937) proved that *streptococci* introduced experimentally into the normal vagina could not be recovered after 21 to 26 hours. The conditions are different during the puerperium. The acid secretion of the vagina is replaced by the slightly alkaline lochia which is favourable to the growth of pathogenic bacteria. The cervical canal is open and the uterus contains a raw area at the site of the separated placenta. These factors make the puerperal uterus peculiarly vulnerable to bacterial infections. The uterine cavity, which is normally sterile, is gradually invaded by different bacteria after delivery. Twenty-five per cent of the cervix are infected on the first day, 50 per cent on the 2nd and 100 per cent on the 3rd day of puerperium, and infection of the uterus following later, reaches its height by the 5th day (Armstrong and Burt-White, 1929).

Although it is now common knowledge that the hæmolytic *streptococcus* is the most dangerous agent in the causation of severe pyrexia after childbirth the incidence of this organism in a series of cases has been investigated by few

The results of the different workers are given in Table I

The hæmolytic *streptococci* isolated by some of the early workers noted in Table I from the cervix of febrile puerpera could not be distinguished from those isolated from afebrile cases by any of the tests at their disposal Hare and Colebrook (1934) could distinguish the majority of the 'saprophytic' strains, but certain strains from afebrile cases behaved biochemically like those isolated from febrile ones Lancefield and Hare (1935) employing the same strains examined by Hare and Colebrook (*loc cit*) distinguished all those strains by the serological method of grouping and discussed their pathogenicity

Certain biochemical tests which have been devised from time to time can also be regarded as useful adjuncts to the serological grouping

In this paper it is intended to show the incidence of hæmolytic *streptococci* from a series of cases of confinement and to determine the pathogenicity of the different groups of these organisms

SOURCE OF STRAIN

Swabs were taken by one of us (excepting on a few occasions when they were taken by trained nurses) on the 3rd or 4th day of the puerperium from consecutive cases delivered in the Hospital for Women, Medical College, Patna A separate speculum of the Fergusson type was used for each case The vulva was wiped with Dettol cream and the speculum introduced carefully without touching the external genitalia as far as possible Care was taken that no antiseptic was introduced into the vaginal canal Swabs were taken by touching the external os of the uterine cervix or the vaginal vault

ISOLATION OF THE STRAINS AND EXPERIMENTAL PROCEDURE

The same technique and tests as described in the previous paper (Chatterjee and Mitter, *loc cit*) were followed

RESULTS

From Table II it will be seen that out of 61 strains isolated from 130 patients 21 strains belonged to group A, 8 to group B, 1 to group C, 9 to group D, 16 to group G and 2 to group K No strain belonging to groups E, F and H was isolated The groupings have been done entirely on the basis of precipitin test Four strains could not be placed in any of the groups due to variable results

TABLE I

Author	Location in hospital (H) or in patient's home (H)	Number of patients examined	Type of examination	Pathologic examination from biopsy	Method of treatment	Outcome in 100 cases (per cent)
Payson and Wright (1930)	H	1114	Before biopsy	1114 (100 per cent)		
Rose (1933)	H	1,344	Autopsy	1,344 (100 per cent)		
Rose (1934)	H	1,050	On admission	1,050 (100 per cent)		40
Rose (1935)	H	1,050	During 2nd stage	1,050 (100 per cent)	13*	40
Rose (1935)	H	1,050	During preoperative	1,050 (100 per cent)	13*	40
Rose and Goldbrook (1935)	H	956	At onset of labor	956 (100 per cent)	1	42
Rose and Goldbrook (1935)	H	957	On day 15th day of postpartum	957 (100 per cent)	1	40

* The severity of infection not specified.

TABLE II

Number of strains	PRECIPITATION WITH SERA OF GROUP										Formation of soluble hemolysin	Final pH in 1 per cent dextrose broth	Reduction of methylene blue in milk	GROWTH IN		FERMENTATION OF					
														10 per cent bile agar	40 per cent bile agar	Glucose	Lactose	Salicin	Mannitol	Trehalose	Sorbitol
	A	B	C	D	E	F	G	H	K												
1	+	-	-	-	-	-	-	-	-	++	5.0	-	-	+	+	+	+	+	+		
5	+	-	-	-	-	-	-	-	-	++	4.9-5.2	-	-	+	+	+	+	+	+		
15	+	-	-	-	-	-	-	-	-	++	4.9-5.2	2+	-	+	+	+	+	+	+		
5	-	+	-	-	-	-	-	-	-	++	4.4-4.6	+	-	+	+	+	+	+	+		
1	-	+	-	-	-	-	-	-	-	+	4.5	+	-	+	+	+	+	+	+		
2	-	+	-	-	-	-	-	-	-	++	4.4-4.7	+	-	+	+	+	+	+	+		
1	-	-	+	-	-	-	-	-	-	++	4.9	-	-	+	+	+	+	+	+		
7	-	-	-	+	-	-	-	-	-	-	4.8-5.1	+	+	+	+	+	+	+	+		
2	-	-	-	-	-	-	-	-	-	-	5.0-5.1	+	+	+	+	+	+	+	+		
4	-	-	-	-	-	-	+	-	-	++	4.0-4.8	+	-	+	+	+	+	+	+		
12	-	-	2±	-	-	-	+	-	-	++	4.8-5.1	4+	-	+	+	+	+	+	+		
2	-	-	-	-	-	-	-	-	+	-	5.2-5.3	-	-	-	-	-	-	-	-		
3	-	+	±	+	-	-	+	-	-	++	4.9-5.3	-	-	-	+	+	+	+	+		
1	±	-	±	±	-	-	±	-	-	++	5.0	-	-	+	+	+	+	+	+		
Totals 61	21	8	1	9	-	-	16	-	2												

The last four strains which could not be placed in one or the other group are not included in the totals of group

The result obtained by other workers is appended below in Table III —

TABLE III

Author	Number of cases	Number of strains	Clinical condition of patient	NUMBER OF STRAINS IN EACH SEROLOGICAL GROUP										Unclassified
				A	B	C	D	E	F	G	H	I	K	
Lancefield and Hare (1935)	837	85	Afebrile	1	26	5	26	—	2	3	*	*		3
			Mild fever	—	7	1	8	—	—	1	*	*		1
			Severe infection	1	—	—	—	—	—	—	*	*		Nil
Present series	130	61	Afebrile	†5	7	—	8	—	—	11a	—	—	2	4
			Mild fever	5	—	—	1	—	—	4	—	—	—	Nil
			Severe infection	11	1c	1b	—	—	—	1b	—	—	—	Nil

* Not tested

† Patients observed only for 3 days of puerperium

a One died

b Also overwhelming *Staph aureus* infection

c Malaria parasite was found in the blood

The hæmolysin test gave strongly positive reaction with groups A, C and G. It was variable in group B and negative in groups D and K.

One strain of group A did not ferment lactose and 5 did not ferment salicin.

One strain of group B fermented sorbitol in addition to trehalose.

The only strain of C behaved like a human strain.

Growth in methylene blue and fermentation of mannite were observed in group D strains only.

Comments on the strains belonging to different groups—It is to be noted that most of these were admitted to the hospital as emergency cases and were delivered immediately or a few hours after admission. About one-third of them were examined internally by indigenous 'dais' during labour before their admission in the hospital.

Group A—Out of 130 cases 21 had group A strains. Of these 11 had a febrile puerperium which may be considered severe. Of the rest, 5 had slight or transient pyrexia and the fever never went up higher than 99.8°F. The cases could be followed up to the 6th day of puerperium. The other 5 cases belonging to this group were afebrile till they left the hospital on the 3rd or the 4th day of puerperium and so it was not possible to observe whether the patients had any pyrexia later. It has been observed in one case that the patient had a transient fever on the 4th day.

genital tract by one of the several ways suggested by Colebrook, D (1935) and Colebrook, L (1936)

It is therefore evident that group A *streptococci* if present in the cervix post partum almost always give rise to severe puerperal infection. Group G *streptococci* sometimes cause mild infection. It is improbable that organisms of the other groups are responsible for any such infection in the human beings.

It may be pointed out that as we have now definite knowledge of the source of severe puerperal fever due to hæmolytic *streptococci*, its prevention is a practical proposition.

SUMMARY

- 1 Out of 130 cervical swabs collected post partum from consecutive cases of confinement, 61 yielded hæmolytic *streptococci*
- 2 Cases having severe puerperal pyrexia had group A *streptococcal* infection
- 3 Some strains of hæmolytic *streptococci* from mild puerperal infections of the uterus belonged to group A
- 4 Some of the group G strains caused mild puerperal infections
- 5 Most of the strains isolated from the cervix of women who had afebrile puerperium belonged to groups other than A
- 6 The source of infection has been discussed. The throat of human being has been suggested to be the primary source of these organisms

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A MODIFICATION OF THE ROUTINE DILUTION TESTS AND TABLES SHOWING THE MOST PROBABLE NUMBER OF ORGANISMS AND THE STANDARD ERROR OF THIS NUMBER

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In previous papers (Swaroop, 1938, 1940) formulæ were developed for the purpose of determining the accuracy with which the most probable number of organisms in water samples may be estimated by the so-called dilution tests. It is well known that modifications in carrying out dilution tests can be introduced by changing the number of tubes taken for each dilution or by choosing varying dilutions of the suspected water-supply. It was shown that the accuracy of estimation of the most probable number of organisms depends not only on the number of tubes or on the dilutions chosen but also upon the number of organisms present in unit volume of the supply. Without the knowledge of the most probable number of organisms, which clearly is what the test is designed to estimate, it may appear *prima facie* impossible to lay down any general rules which should govern an analyst in designing his tests. But, in the absence of this knowledge, it was suggested how the test could be usefully planned.

Two sets of dilutions which have been recommended for routine tests by the Ministry of Health, England, (1939) are —

(i) The dilutions $1/2$, $1/10$, $1/100$ with 1, 5, and 5 tubes for the respective dilutions, or

(ii) The dilutions $1/10$, $1/100$, $1/1,000$ with 5 tubes chosen for each dilution.

The efficiency of these two sets of dilutions was discussed and it was pointed out that, in those cases in which the contamination of the suspected supply is not very high, such as piped water-supply, it would be advantageous to replace these two tests by taking a set of dilutions similar to those as in the case (i) above but by choosing an equal number of tubes for each dilution. This modification shows greater efficiency in the estimation of the probable number of organisms over a

range of values for which the test will usually have to be carried out. For instance, it can be shown that, when the number of organisms in 100 c c is one, the accuracy gained by taking 5 tubes of each of the dilutions 1/2, 1/10 and 1/100 is the same as will be obtained by taking 22 tubes of each of the dilutions 1/10, 1/100 and 1/1,000 and that negligible gain in accuracy is produced by choosing 5, 25 and 25 tubes of the dilutions 1/2, 1/10 and 1/100 respectively. Again, if the number of organisms in the supply is 20 per 100 c c the effect of taking one tube of each of the dilutions 1/2, 1/10 and 1/100 is for practical purposes equivalent to taking 1, 5 and 5 of the three dilutions 1/2, 1/10 and 1/100. In other words if the suggested modification was used we would require only 3 tubes instead of 11.

In view of the relative advantages of this modification over the two routine sets now commonly employed and the possibility of their replacement by the suggested modified test, tables are provided, for reading at a glance, the most probable number of organisms in 100 c c of the supply and the standard error of the most probable number when, for each of the three dilutions 1/2, 1/10, and 1/100 the number of tubes chosen is either 2, 3, 5 or 10. An attempt has been made to avoid the introduction of those combinations of positive and negative results, which although a theoretical possibility, are not likely to arise in actual practice.

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TABLE I

Showing the most probable number of organisms present in 100 c c of water and the standard error of the most probable number for various combinations of positive and negative results when 2 tubes are used for each of the quantities 50 c c, 10 c c and 1 c c

NUMBER OF TUBES GIVING POSITIVE REACTION OUT OF			Most probable number of organisms present in 100 c c	Standard error of the most probable number of organisms	NUMBER OF TUBES GIVING POSITIVE REACTION OUT OF			Most probable number of organisms present in 100 c c	Standard error of the most probable number of organisms
2 tubes, each of 50 c c	2 tubes, each of 10 c c	2 tubes, each of 1 c c			2 tubes, each of 50 c c	2 tubes, each of 10 c c	2 tubes, each of 1 c c		
0	0	1	1	1	0	1	1	2	2
0	0	2	2	2	0	1	2	3	2
0	1	0	1	1	0	2	0	2	2

TABLE I—concl'd

NUMBER OF TUBES GIVING POSITIVE REACTION OUT OF			Most probable number of organisms present in 100 c c	Standard error of the most probable number of organisms	NUMBER OF TUBES GIVING POSITIVE REACTION OUT OF			Most probable number of organisms present in 100 c c	Standard error of the most probable number of organisms
2 tubes, each of 50 c c	2 tubes, each of 10 c c	2 tubes, each of 1 c c			2 tubes, each of 50 c c	2 tubes, each of 10 c c	2 tubes, each of 1 c c		
0	2	1	3	2	1	2	2	7	5
0	2	2	4	3	2	0	0	4	3
1	0	0	1	1	2	0	1	6	4
1	0	1	2	2	2	0	2	10	8
1	0	2	3	2	2	1	0	7	5
1	1	0	2	2	2	1	1	13	10
1	1	1	4	3	2	1	2	21	17
1	1	2	5	4	2	2	0	24	19
1	2	0	4	3	2	2	1	70	68
1	2	1	5	4					

TABLE II

Showing the most probable number of organisms present in 100 c c of water and the standard error of the most probable number for various combinations of positive and negative results when 3 tubes are used for each of the quantities 50 c c, 10 c c and 1 c c

NUMBER OF TUBES GIVING POSITIVE REACTION OUT OF			Most probable number of organisms present in 100 c c	Standard error of the most probable number of organisms	NUMBER OF TUBES GIVING POSITIVE REACTION OUT OF			Most probable number of organisms present in 100 c c	Standard error of the most probable number of organisms
3 tubes, each of 50 c c	3 tubes, each of 10 c c	3 tubes each of 1 c c			3 tubes, each of 50 c c	3 tubes, each of 10 c c	3 tubes, each of 1 c c		
0	0	1	1	1	0	0	3	2	1
0	0	2	1	1	0	1	0	1	1

TABLE II—*contd*

NUMBER OF TUBES GIVING POSITIVE REACTION OUT OF			Most probable number of organisms present in 100 c c	Standard error of the most probable number of organisms	NUMBER OF TUBES GIVING POSITIVE REACTION OUT OF			Most probable number of organisms present in 100 c c	Standard error of the most probable number of organisms
3 tubes, each of 50 c c	3 tubes, each of 10 c c	3 tubes, each of 1 c c			3 tubes, each of 50 c c	3 tubes, each of 10 c c	3 tubes, each of 1 c c		
0	1	1	1	1	1	3	1	4	2
0	1	2	2	1	1	3	2	5	3
0	1	3	2	1	1	3	3	5	3
0	2	0	1	1	2	0	1	3	2
0	2	1	2	1	2	0	2	3	2
0	2	2	2	1	2	0	3	4	2
0	2	3	3	2	2	1	0	3	2
0	3	0	2	1	2	1	1	4	2
0	3	1	2	1	2	1	2	5	3
0	3	2	3	2	2	1	3	6	4
0	3	3	4	2	2	2	0	4	2
1	0	0	1	1	2	2	1	5	3
1	0	1	1	1	2	2	2	6	4
1	0	2	2	1	2	2	3	7	4
1	0	3	3	2	2	3	0	5	3
1	1	0	2	1	2	0	0	2	1
1	1	1	2	1	2	3	1	7	4
1	1	2	3	2	2	3	2	8	5
1	1	3	4	2	2	3	3	10	6
1	2	0	2	1	3	0	0	4	2
1	2	1	3	2	3	0	1	5	3
1	2	2	4	2	3	0	2	7	4
1	2	3	4	2	3	0	3	10	6
1	3	0	3	2	3	1	0	6	4

TABLE II—*concl'd*

NUMBER OF TUBES GIVING POSITIVE REACTION OUT OF			Most probable number of organisms present in 100 c c	Standard error of the most probable number of organisms	NUMBER OF TUBES GIVING POSITIVE REACTION OUT OF			Most probable number of organisms present in 100 c c	Standard error of the most probable number of organisms
3 tubes, each of 50 c c	3 tubes, each of 10 c c	3 tubes, each of 1 c c			3 tubes, each of 50 c c	3 tubes, each of 10 c c	3 tubes, each of 1 c c		
3	1	1	8	5	3	2	2	21	13
3	1	2	12	8	3	2	3	29	19
3	1	3	18	10	3	3	0	24	16
3	2	0	10	6	3	3	1	46	35
3	2	1	15	10	3	3	2	110	82

TABLE III

Showing the most probable number of organisms present in 100 c c of water and the standard error of the most probable number for various combinations of positive and negative results when 5 tubes are used for each of the quantities 50 c c, 10 c c and 1 c c

NUMBER OF TUBES GIVING POSITIVE REACTION OUT OF			Most probable number of organisms present in 100 c c	Standard error of the most probable number of organisms	NUMBER OF TUBES GIVING POSITIVE REACTION OUT OF			Most probable number of organisms present in 100 c c	Standard error of the most probable number of organisms
5 tubes, each of 50 c c	5 tubes, each of 10 c c	5 tubes, each of 1 c c			5 tubes, each of 50 c c	5 tubes, each of 10 c c	5 tubes, each of 1 c c		
0	0	1	1	1	0	2	1	1	1
0	0	2	1	1	0	3	0	1	1
0	1	0	1	1	1	0	0	1	1
0	1	1	1	1	1	0	1	1	1
0	1	2	1	1	1	0	2	1	1
0	2	0	1	1	1	0	3	2	1

TABLE III—*contd*

NUMBER OF TUBES GIVING POSITIVE REACTION OUT OF			Most probable number of organisms present in 100 c c	Standard error of the most probable number of organisms	NUMBER OF TUBES GIVING POSITIVE REACTION OUT OF			Most probable number of organisms present in 100 c c	Standard error of the most probable number of organisms
5 tubes, each of 50 c c	5 tubes, each of 10 c c	5 tubes, each of 1 c c			5 tubes, each of 50 c c	5 tubes, each of 10 c c	5 tubes, each of 1 c c		
1	1	0	1	1	3	0	2	2	1
1	1	1	1	1	3	1	0	2	1
1	1	2	2	1	3	1	1	2	1
1	2	0	1	1	3	1	2	3	1
1	2	1	2	1	3	1	3	4	2
1	2	2	2	1	3	2	0	3	1
1	3	0	2	1	3	2	1	3	1
1	3	1	2	1	3	2	2	4	2
1	4	0	2	1	3	3	0	3	1
2	0	0	1	1	3	3	1	4	2
2	0	1	1	1	3	4	0	4	2
2	0	2	2	1	3	4	1	4	2
2	0	3	2	1	3	5	0	5	2
2	1	0	1	1	4	0	0	2	1
2	1	1	2	1	4	0	1	3	1
2	1	2	2	1	4	0	2	3	1
2	2	0	2	1	4	0	3	4	2
2	2	1	2	1	4	1	0	3	1
2	2	2	3	1	4	1	1	4	2
2	3	0	2	1	4	1	2	4	2
2	3	1	3	1	4	2	0	4	2
2	4	0	3	1	4	2	1	4	2
3	0	0	2	1	4	2	2	5	2
3	0	1	2	1	4	3	0	5	2

TABLE III—*concd*

NUMBER OF TUBES GIVING POSITIVE REACTION OUT OF			Most probable number of organisms present in 100 c c	Standard error of the most probable number of organisms	NUMBER OF TUBES GIVING POSITIVE REACTION OUT OF			Most probable number of organisms present in 100 c c	Standard error of the most probable number of organisms
5 tubes each of 50 c c	5 tubes, each of 10 c c	5 tubes each of 1 c c			5 tubes, each of 50 c c	5 tubes, each of 10 c c	5 tubes, each of 1 c c		
4	3	1	5	2	5	2	4	15	7
4	3	2	6	3	5	2	5	18	9
4	4	0	6	3	5	3	0	9	4
4	4	1	7	3	5	3	1	11	5
4	5	0	7	3	5	3	2	14	7
4	5	1	8	4	5	3	3	18	9
5	0	0	4	2	5	3	4	21	10
5	0	1	4	2	5	3	5	25	13
5	0	2	6	3	5	4	0	13	6
5	0	3	7	3	5	4	1	17	8
5	0	4	8	4	5	4	2	22	11
5	1	0	5	2	5	4	3	28	14
5	1	1	6	3	5	4	4	35	10
5	1	2	7	3	5	4	5	43	25
5	1	3	9	4	5	5	0	24	12
5	2	0	6	3	5	5	1	35	19
5	2	1	8	4	5	5	2	54	33
5	2	2	10	5	5	5	3	92	55
5	2	3	12	6	5	5	4	161	89

TABLE IV.

Showing the most probable number of organisms present in 100 c c of water and the standard error of the most probable number for various combinations of positive and negative results when 10 tubes are used for each of the quantities 50 c c, 10 c c and 1 c c

NUMBER OF TUBES GIVING POSITIVE REACTION OUT OF			Most probable number of organisms present in 100 c c	Standard error of the most probable number of organisms	NUMBER OF TUBES GIVING POSITIVE REACTION OUT OF			Most probable number of organisms present in 100 c c	Standard error of the most probable number of organisms
10 tubes, each of 50 c c	10 tubes, each of 10 c c	10 tubes, each of 1 c c			10 tubes, each of 50 c c	10 tubes, each of 10 c c	10 tubes, each of 1 c c		
1	0	0	1	* <0.5	5	0	0	1	<0.5
1	1	0	1	<0.5	5	1	0	1	<0.5
1	1	1	1	<0.5	5	2	0	2	1
2	0	0	1	<0.5	5	2	1	2	1
2	1	0	1	<0.5	5	3	0	2	1
2	1	1	1	<0.5	5	3	1	2	1
2	2	1	1	<0.5	5	3	2	2	1
3	0	0	1	<0.5	5	3	3	3	1
3	1	0	1	<0.5	6	0	0	2	1
3	1	1	1	<0.5	6	1	0	2	1
3	2	1	1	<0.5	6	1	1	2	1
3	3	2	2	1	6	2	0	2	1
3	3	0	1	<0.5	6	3	0	2	1
4	0	0	1	<0.5	6	3	1	3	1
4	1	0	1	<0.5	6	3	2	3	1
4	2	0	1	<0.5	6	2	1	2	1
4	3	1	2	1	6	4	1	3	1
4	2	1	2	1	6	4	2	3	1
4	3	0	2	1	6	4	3	3	1

* < sign denotes less than the number following the sign.

TABLE IV—*contd*

NUMBER OF TUBES GIVING POSITIVE REACTION OUT OF			Most probable number of organisms present in 100 c.c.	Standard error of the most probable number of organisms	NUMBER OF TUBES GIVING POSITIVE REACTION OUT OF			Most probable number of organisms present in 100 c.c.	Standard error of the most probable number of organisms
10 tubes, each of 50 c.c.	10 tubes, each of 10 c.c.	10 tubes, each of 1 c.c.			10 tubes, each of 50 c.c.	10 tubes, each of 10 c.c.	10 tubes, each of 1 c.c.		
6	4	0	3	1	8	0	0	2	1
6	5	0	3	1	8	1	0	3	1
6	5	1	3	1	8	1	1	3	1
6	5	2	3	1	8	1	2	3	1
6	5	3	4	1	8	2	0	3	1
7	0	0	2	1	8	2	1	3	1
7	1	0	2	1	8	2	2	4	1
7	1	1	2	1	8	3	0	3	1
7	2	0	2	1	8	3	1	4	1
7	2	1	3	1	8	3	2	4	1
7	2	2	3	1	8	3	3	4	1
7	3	0	3	1	8	4	0	4	1
7	3	1	3	1	8	4	1	4	1
7	3	2	3	1	8	4	2	4	1
7	4	0	3	1	8	4	3	5	2
7	4	1	3	1	8	4	4	5	2
7	4	2	4	1	8	5	0	4	1
7	4	3	4	1	8	5	1	5	2
7	4	4	4	1	8	5	2	5	2
7	5	0	3	1	8	5	3	5	2
7	5	1	4	1	8	5	4	6	2
7	5	2	4	1	8	5	5	6	2
7	5	3	4	1	8	5	6	7	2
7	5	4	5	2	8	6	0	5	2

Routine Dilution Tests

TABLE IV—contd

NUMBER OF TUBES GIVING POSITIVE REACTION OUT OF			Most probable number of organisms present in 100 c c	Standard error of the most probable number of organisms	NUMBER OF TUBES GIVING POSITIVE REACTION OUT OF			Most probable number of organisms present in 100 c c	Standard error of the most probable number of organisms
10 tubes, each of 50 c c	10 tubes, each of 10 c c	10 tubes, each of 1 c c			10 tubes, each of 50 c c	10 tubes, each of 10 c c	10 tubes, each of 1 c c		
8	6	1	5	2	9	0	0	3	1
8	6	2	5	2	9	1	0	3	1
8	6	3	6	2	9	2	0	4	1
8	6	4	6	2	9	2	1	4	1
8	6	5	7	2	9	3	2	5	2
8	6	6	7	2	9	4	0	5	2
8	6	7	8	3	9	4	1	5	2
8	7	0	5	2	9	4	2	6	2
8	7	1	6	2	9	4	3	6	2
8	7	2	6	2	9	5	0	5	2
8	7	3	7	2	9	5	1	6	2
8	7	4	7	2	9	5	2	6	2
8	7	5	8	3	9	5	3	7	2
8	7	6	8	3	9	5	4	8	3
8	7	7	9	3	9	5	5	8	3
8	8	0	6	2	9	6	0	6	2
8	8	1	6	2	9	6	1	7	2
8	8	2	7	2	9	6	2	7	2
8	8	3	7	2	9	6	3	8	3
8	8	4	8	3	9	6	4	9	3
8	8	5	8	3	9	6	5	9	3
8	8	6	9	3	9	6	6	10	3
8	8	7	10	3	9	7	0	7	2
8	8	8	10	3	9	7	1	7	2

TABLE IV—contd

NUMBER OF TUBES GIVING POSITIVE REACTION OUT OF			Most probable number of organisms present in 100 c c	Standard error of the most probable number of organisms	NUMBER OF TUBES GIVING POSITIVE REACTION OUT OF			Most probable number of organisms present in 100 c c	Standard error of the most probable number of organisms
10 tubes, each of 50 c c	10 tubes, each of 10 c c	10 tubes, each of 1 c c			10 tubes each of 50 c c	10 tubes, each of 10 c c	10 tubes, each of 1 c c		
9	7	2	8	3	10	1	0	4	1
9	7	3	9	3	10	2	0	5	2
9	7	4	10	3	10	2	1	5	2
9	7	5	11	4	10	3	2	7	3
9	7	6	11	4	10	4	2	8	3
9	8	0	8	3	10	5	2	9	3
9	8	1	8	3	10	5	3	10	3
9	8	2	9	3	10	5	4	12	4
9	8	3	10	3	10	6	2	11	4
9	8	4	11	4	10	6	3	13	5
9	8	5	12	4	10	6	4	14	5
9	8	6	13	5	10	6	5	16	6
9	8	7	14	5	10	7	2	14	5
9	9	0	9	3	10	7	3	16	6
9	9	1	10	3	10	7	4	17	6
9	9	2	11	4	10	7	5	19	7
9	9	3	12	4	10	7	6	22	8
9	9	4	13	5	10	8	2	17	6
9	9	5	14	5	10	8	3	20	7
9	9	6	15	5	10	8	4	22	8
9	9	7	16	6	10	8	5	25	9
9	9	8	17	6	10	8	6	28	10
9	9	9	19	7	10	9	3	26	9
10	0	0	4	1	10	9	4	30	11

Routine Dilution Tests

TABLE IV—concl'd

NUMBER OF TUBES GIVING POSITIVE REACTION OUT OF			Most probable number of organisms present in 100 c c	Standard error of the most probable number of organisms	NUMBER OF TUBES GIVING POSITIVE REACTION OUT OF			Most probable number of organisms present in 100 c c	Standard error of the most probable number of organisms
10 tubes, each of 50 c c	10 tubes, each of 10 c c	10 tubes, each of 1 c c			10 tubes, each of 50 c c	10 tubes, each of 10 c c	10 tubes, each of 1 c c		
10	9	5	35	13	10	10	5	70	30
10	9	6	40	16	10	10	6	92	39
10	9	7	46	19	10	10	7	120	48
10	10	3	43	18	10	10	8	161	63
10	10	4	54	23	10	10	9	230	95

A CONSIDERATION OF THE ACCURACY OF ESTIMATION OF THE MOST PROBABLE NUMBER OF ORGANISMS BY DILUTION TEST

BY

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[Received for publication, January 27, 1941]

THE commonly employed method of assessing the potability of water is to estimate the most probable number of organisms present in fixed quantities of the supply by means of the so-called dilution tests. A number of dilutions of the original sample is prepared and each is inoculated in a suitable medium. The presence or absence of organisms is then judged by the evidence of gas formation. Usually 5 or 10 tubes are taken for each dilution and the numbers of tubes showing positive or negative reactions are made the basis for the calculation of the most probable number of organisms present in the sample.

In two papers Halvorson and Ziegler (1933) have attempted to build up a statistical technique for appraising the accuracy of the dilution method. In their first paper (Part II) they considered a simple case when only one dilution was chosen and came to the conclusion that the accuracy of estimation depends not only upon the number of tubes inoculated from the sample but also upon the bacterial density of the suspected supply. This result is in agreement with that arrived at by other workers in the field, viz Greenwood and Yule (1917), McCrady (1915) and Stein (1919).

Dilution tests are, however, carried out by taking for each test a number of dilutions. In order to meet these cases Halvorson and Ziegler extended their analysis and came to the conclusion that 'when three effective dilutions are used to determine the bacterial population, the accuracy is independent of the number of organisms and depends only on the number of tubes used in each dilution'. The same problem was recently studied by the writer (Swaroop, 1938) and a general formula was given for calculating the standard error of the most probable number of organisms. While discussing the efficiency of the dilution method, on the basis of this formula, it was stated that the accuracy of the dilution technique was dependent on all the relevant factors, viz (i) the number of tubes used

where s_x stands for the number of tubes chosen for each dilution, S denotes the sum for all the dilutions, a is the dilution factor, x the stage of dilution and E_x stands for the expression—

$$1 / e^{n/a^x} - 1$$

e being the usual Napierian e With the help of this expression for σ_n we may calculate the coefficient of variation for any dilution and for any bacterial suspension But when it is remembered that, in testing the potability of water, in actual practice the test will have usually to be carried out over samples which do not contain more than 10 organisms per 100 c c and certainly not for those containing more than 100 per 100 c c , it becomes necessary to examine in detail the coefficients of variations for all values up to 10 Table I sets out the values of coefficients of variation for all numbers of n up to 20 and then at intervals of ten up to 100 These values relate to cases when only single dilutions are taken each time and only one tube is used

TABLE I

*Showing coefficients of variation for varying values of the most probable number when different single dilutions are taken
The number of tubes taken is one in each case.*

Most probable number per 100 c c	COEFFICIENT OF VARIATION WHEN ONE TUBE IS TAKEN FOR EACH DILUTION —			
	1/2	1/10	1/100	1/1,000
1	161	324	1,003	1,000*
2	131	235	711	1,000*
3	124	197	582	1,000*
4	126	175	505	1,000*
5	134	161	453	1,000*
6	146	151	414	1,000*
7	162	144	385	1,000*
8	183	138	361	1,000*
9	210	134	341	1,000*
10	243	131	324	1,000*

* denotes greater than 1,000

TABLE I—concl'd

Most probable number per 100 c c	COEFFICIENT OF VARIATION WHEN ONE TUBE IS TAKEN FOR EACH DILUTION —			
	1/2	1/10	1/100	1/1,000
11	284	129	310	958
12	334	127	298	916
13	397	126	287	880
14	473	125	277	848
15	567	124	268	820
16	682	124	260	794
17	824	124	253	770
18	1,000*	125	247	749
19	1,000*	126	241	729
20	1,000*	126	235	711
30	1,000*	146	197	582
40	1,000*	183	175	505
50	1,000*	243	161	453
60	1,000*	334	151	414
70	1,000*	473	144	385
80	1,000*	682	138	361
90	1,000*	1,000*	134	341
100	1,000*	1,000*	131	324

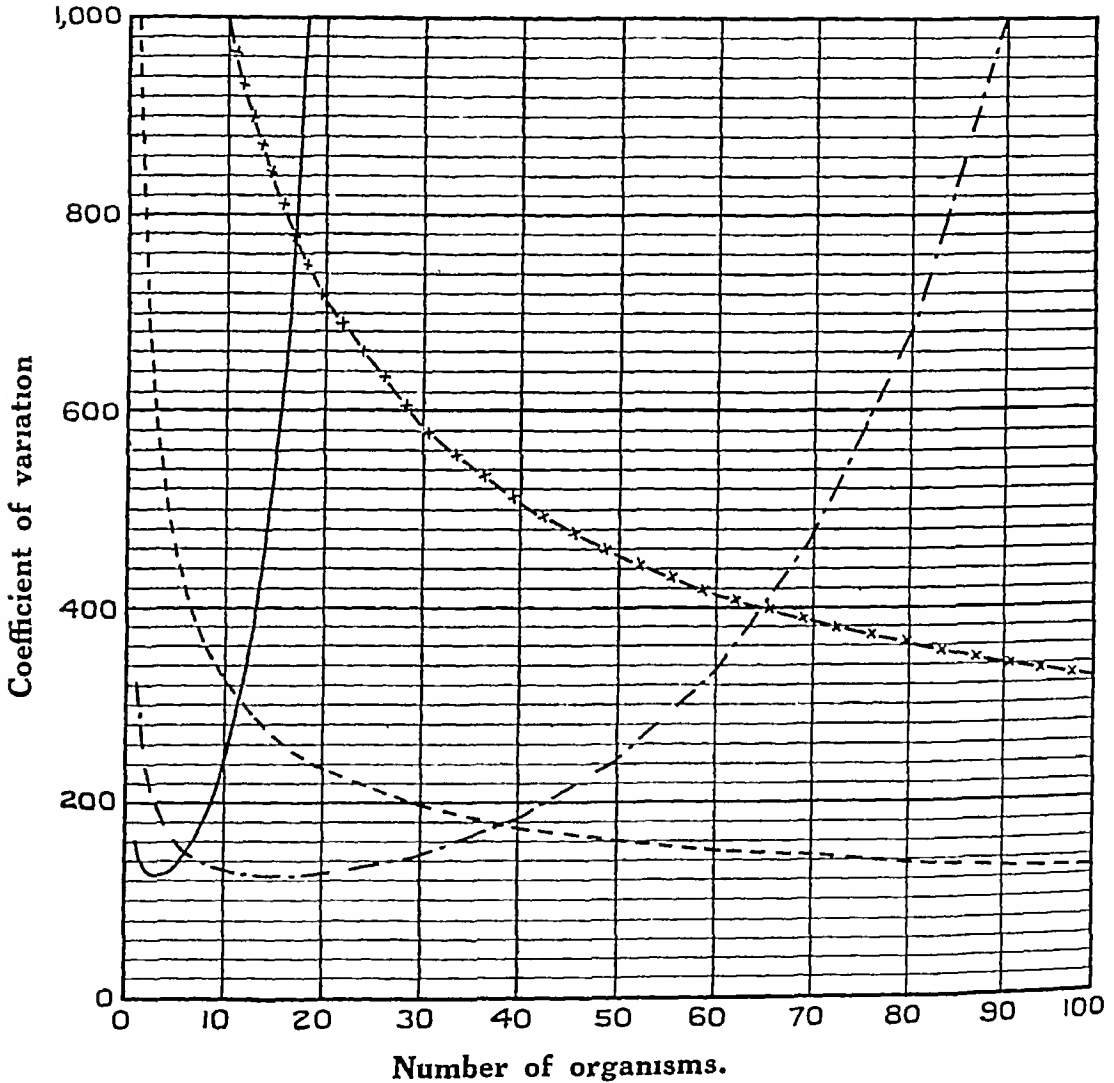
* Denotes greater than 1,000

Graph 1 constructed with the help of the values of coefficients of variation in Table I shows the curves for each dilution separately. It may be mentioned that the nearer is the curve to the abscissal axis the better is it from the point of view of accuracy. The dilution 1/2 is most efficient for very low values of the probable number. If an arbitrary line is drawn at value 200 for coefficients of variation and values below it are taken as efficient it may be seen that the dilution 1/2 is efficient up to $n=8$. The efficiency of dilution 1/10 begins at $n=3$ and continues up to $n=40$. The efficiency of dilution 1/100 begins

after $n = 30$ and continues up to the range studied in this paper. The dilution $1/1,000$ may be considered to be inefficient as judged by the above standard.

GRAPH 1

Curves of coefficient of variation for varying values of the probable number of organisms when only one dilution is taken for each test



DILUTIONS

 $\frac{1}{2}$ ----- $\frac{1}{10}$ - - - - - $\frac{1}{100}$ $\frac{1}{1,000}$ - - - x - - -

Table II sets out the values of the coefficients of variation when combinations of dilutions are taken for each test. Since the accuracy depends upon the number of tubes chosen for each dilution an attempt has been made to keep in each test the same number of tubes. Four different dilution sets are studied, viz —

- (i) Dilutions 1/2 and 1/10 and the tubes taken being 2 and 10 respectively
- (ii) Dilutions 1/2, 1/10 and 1/100 and the tubes taken being 1, 5 and 5 respectively
- (iii) Dilutions 1/10, 1/100 and 1/1,000 and the tubes taken being 4 in each case
- (iv) Dilutions 1/2, 1/10 and 1/100 and the tubes taken being 4 in each case

It may thus be seen that the total number of tubes taken is 12 in each case except in the case of (iii) when the number of tubes taken is only one less, i.e. eleven

TABLE II

Showing coefficients of variation for different values of the most probable number when varying sets of dilutions are taken

Most probable number of organisms per 100 c.c.	COEFFICIENTS OF VARIATION FOR							
	Dilutions Tubes		Dilutions Tubes		Dilutions Tubes		Dilutions Tubes	
	1/2 1/10	2 10	1/2 1/10 1/100	1 5 5	1/10 1/100 1/1,000	4 4 4	1/2 1/10 1/100	4 4 4
1	76		105		154		71	
2	58		79		111		57	
3	51		69		93		52	
4	47		64		82		50	
5	45		61		75		50	
6	43		58		71		51	
7	42		57		67		52	
8	41		55		64		53	
9	41		54		62		54	
10	40		53		60		54	
11	40		52		59		55	

TABLE II—*concl'd*

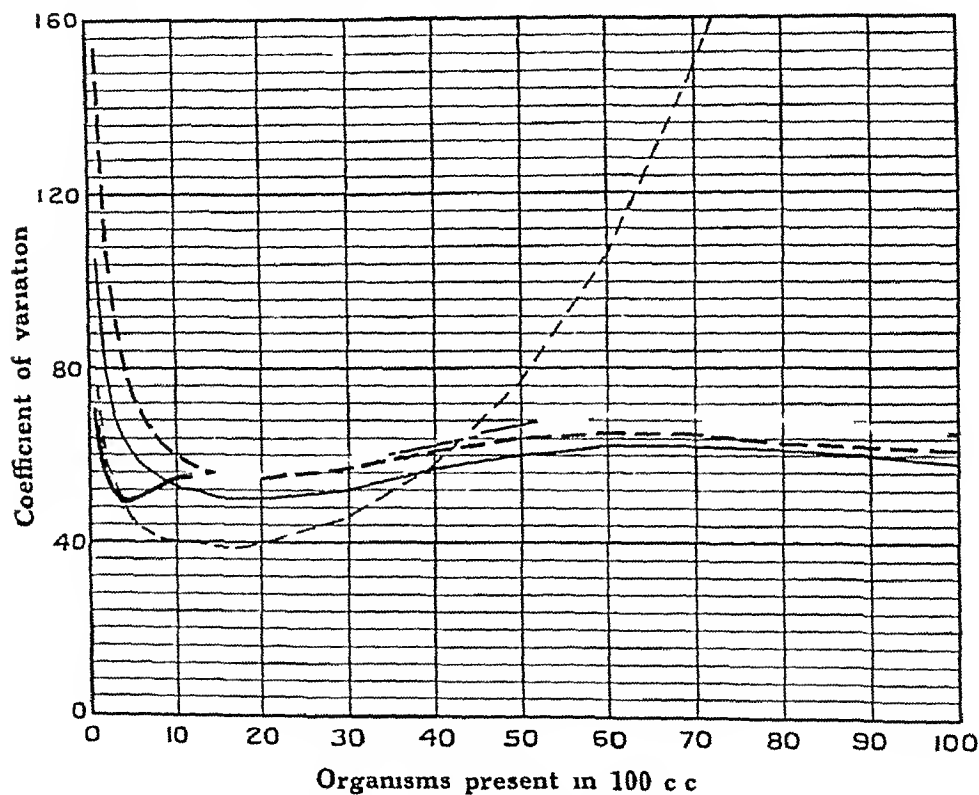
Most probable number of organisms per 100 c c	COEFFICIENTS OF VARIATION FOR							
	Dilutions	Tubes	Dilutions	Tubes	Dilutions	Tubes	Dilutions	Tubes
	$\frac{1}{2}$	2	$\frac{1}{2}$	1	$\frac{1}{10}$	$\frac{4}{5}$	$\frac{1}{2}$	$\frac{4}{5}$
	$\frac{1}{10}$	$\frac{10}{5}$	$\frac{1}{10}$	$\frac{5}{5}$	$\frac{1}{100}$	$\frac{4}{5}$	$\frac{1}{100}$	$\frac{4}{5}$
12	40		52		58		55	
13	39		51		57		55	
14	39		51		56		55	
15	39		50		56		55	
16	39		50		56		55	
17	39		50		55		55	
18	39		50		55		55	
19	40		50		55		55	
20	40		50		55		56	
30	46		52		57		59	
40	58		57		61		62	
50	77		60		64		67	
60	106		62		65		69	
70	149		62		65		69	
80	216		61		63		68	
90	317		60		62		67	
100	471		58		61		65	

Graph 2 shows how the coefficient of variation varies with bacterial density in the case of different sets of dilutions. If the dilution test is carried out with $\frac{1}{2}$ and $\frac{1}{10}$ dilutions and with 2 and 10 tubes respectively the value of the coefficient of variation is 76 when the value of n is unity. With an increase in the value of n the coefficient of variation shows a decrease till the value of n equals 20. After this value of n the curve takes a steep course upwards and it is clear that from a practical point of view this dilution is useless for higher values of n . The curve for the dilutions $\frac{1}{2}$, $\frac{1}{10}$ and $\frac{1}{100}$ with 1, 5 and 5 tubes respectively begins with a high figure when n is unity. After a gradual decline the curve runs a course

which is for practical purposes parallel to the abscissal axis. This dilution although giving a large coefficient of variation for small values on n may be considered to be satisfactory for larger values of the bacterial density.

GRAPH 2

Curves of coefficients of variation for four different sets of dilutions



DILUTIONS	NUMBER OF TUBES OF RESPECTIVE DILUTIONS
$\frac{1}{2}, \frac{1}{10}$ - - - - -	2, 10 - - - - -
$\frac{1}{2}, \frac{1}{10}, \frac{1}{100}$ - - - - -	4, 4, 4 - - - - -
$\frac{1}{10}, \frac{1}{100}, \frac{1}{1000}$ - - - - -	4, 4, 4 - - - - -
$\frac{1}{2}, \frac{1}{10}, \frac{1}{100}$ - - - - -	1, 5, 5 - - - - -

The dilutions 1/10, 1/100 and 1/1,000 with four tubes in each case comprise a set on which Halvorson and Ziegler demonstrated the independence of the coefficient of variation to n . In this case the curve begins at the highest point in comparison with the curves corresponding to the other dilution sets considered. It shows a

fall with increasing values of n and for values of n up to 20 this curve occupies the highest position. For larger values of n , however, the curve has for practical purposes a constant coefficient of variation. The values of the coefficient of variation, when the number of organisms in 100 c c of the supply is 25, 50 or 150, are of about the same order. It is the constancy of coefficient of variation for these large values of n , on the basis of which, they were led to the conclusion that the accuracy of dilution data is independent of the bacterial density. But, as is clear from the graph, it is hardly true that, in the case of those values of n for which the test has usually to be carried out, viz the small values of n , the coefficient of variation is independent of the bacterial density. The coefficient of variation, for instance, shows a decrease from 154 (when n is unity) to 55 (when n is 20).

From the practical point of view the choice set of dilutions should be such as will show only negligible variation in the values of the coefficient of variation over that range of values of bacterial density which usually arise in routine bacteriological examinations. It may be seen from the graph that among the different sets discussed in this paper this condition is approached by the set of dilutions 1/2, 1/10 and 1/100 when the number of tubes is the same for each dilution, viz four. The curve corresponding to this set shows low values of the coefficient of variation for small values of n and keeps to the same order of height as the curves for other dilution sets for higher values of n . Table III sets out the range of variation of the coefficient of variation over the values of n ranging from unity to 100 —

TABLE III

Showing the range of coefficient of variation for $n = 1$ to $n = 100$

Dilution sets	Limits of variation	Range of variation
1/2 and 1/10 with 2 and 10 tubes respectively	471 to 39	432
1/2, 1/10 and 1/100 with 1, 5 and 5 tubes respectively	105 to 50	55
1/10, 1/100 and 1/1,000 with 4 tubes in each case	154 to 55	99
1/2, 1/10 and 1/100 with 4 tubes in each case	71 to 50	21

The comparative stability of the last dilution set is clear from a perusal of the last column of Table III. It is, therefore, suggested that in routine bacteriological examinations of water this dilution set may replace those now commonly employed. In planning the test it will be fairly accurate to assume that this set of dilutions yields a coefficient of variation of the order of 60 when four tubes are chosen for

each dilution. It has been shown in a previous paper that a reduction in coefficient of variation occurs in the same ratio as the square of the number of tubes used. If, therefore, for this set of dilutions, a coefficient of variation as low as y is desired the number of tubes to be taken for each dilution may be calculated from the following simple equation —

The number of tubes to be taken for each dilution in order to get the coefficient of variation as low as $y = 14,400/y^2$

Thus, if it is desired that the standard error should be only 10 per cent of the most probable number the value of $y = 10$ and the number of tubes to be taken for the test is $14,400/10^2$ or 144. The test should therefore be carried out with 144 tubes for each of the three dilutions 1/2, 1/10 and 1/100. Tables which give the most probable number of organisms for various combinations of positive and negative results and the standard error of the most probable number have been published (Swaroop, 1941) for cases when for each dilution either 2, 3, 5 or 10 tubes are used.

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COMPLEMENT-FIXATION BY LEPROUS SERA AFTER ABSORPTION BY VARIOUS ACID-FAST BACILLI

BY

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INTRODUCTION

In a previous publication (Dharmendra and Bose, 1941), it was pointed out that of the antigens prepared from the six different acid-fast bacilli (four isolated from leprous material and *Mycobacterium tuberculosis* and *Mycobacterium phlei*) the one prepared from Lleras' bacillus (isolated by Llera from leprous material) appeared to be slightly more sensitive, since a slightly higher number of leprous sera fixed complement in its presence, and dilution did not reduce complement-fixation with this antigen to the same extent as with the other antigens. This greater sensitivity was, however, not considered to be caused by any specificity as it was seen in cases of leprosy and other diseases also. This question has been further studied by testing the complement-fixing power of leprous sera before and after absorption with the different acid-fast bacilli.

METHODS USED

The method of the preparation of the antigens and the technique of performing the tests have been the same as described previously. Absorption of the sera was carried out by mixing them with thick bacillary suspensions, allowing the mixture to stand overnight and then filtering through a candle. Five different bacillary suspensions were used, these were prepared from cultures of *Myco phlei* and the so-called leprosy bacilli of Lleras, Bayon and Duval and from a leprous nodule. Each serum was divided into six portions, one portion was kept unabsorbed, and the other five portions were absorbed by the above five bacillary suspensions. The

unabsorbed portion and the portions absorbed by the different bacillary suspensions were then tested for their complement-fixing power in presence of all the six antigens. All these sera were tested in 1/5, 1/25, 1/50, 1/100 and 1/200 dilutions.

RESULTS

Sera from 12 cases of the lepromatous type of leprosy were used in this investigation. In a 1/5 dilution the absorbed sera did not differ in any way from the unabsorbed specimens in their complement-fixing power in the presence of any of the six antigens used. In higher dilutions, however, the complement-fixing power of the absorbed sera was less than that of the unabsorbed specimens. This diminution in the complement-fixing power first appeared in the following dilutions, 1/25 in four sera, 1/100 in five sera and 1/200 in three sera.

All the bacillary suspensions used for absorbing the sera acted in a similar way. A serum absorbed with any one of these suspensions showed a reduction in its complement-fixing power not only in the presence of the homologous antigen but also in the presence of the antigens prepared from the other acid-fast bacilli.

ABSORPTION OF THE SERA WITH *staphylococcus* CULTURES

In order to find out whether the absorption of the leprosy sera with bacilli other than those of the acid-fast group will reduce the complement-fixing power of the sera, sera from five cases of the lepromatous type of leprosy were absorbed with cultures of *Staphylococcus aureus*. Complement-fixation by these sera in the presence of the six antigens previously mentioned was tested before and after absorption.

In three of the five sera absorption with *staphylococcus* did not reduce their complement-fixing power in the dilutions tested (1 in 1/200, 1 in 1/100 and 1 in 1/25). In the other two sera absorption with *staphylococcus* reduced to some extent the complement-fixing power. This reduction was first observed in one serum in a 1 in 100 dilution and in the other in a 1 in 25 dilution.

CONCLUSIONS

Complement-fixation tests by leprosy sera absorbed with the different acid-fast bacilli including the so-called leprosy bacilli of Lleras, Duval and Bayon and Hansen's bacilli obtained from a leprosy nodule do not afford any proof of the specificity of any of the cultures from which the antigens had been made.

This confirms the belief that the slightly higher sensitivity of the antigen prepared from Lleras' bacillus is not caused by any specificity. This conclusion receives support from the results of intradermal tests with lepromin (prepared from leprosy nodules) and with material prepared from cultures of Lleras' bacillus.*

* Intradermal injections of lepromin give rise to a nodular reaction in cases of the neural type of leprosy; in cases of the lepromatous type the injection causes a little or no such reaction. The material prepared from cultures of Lleras' bacilli gave positive results in cases of both the 'neural' and the lepromatous type, like the lepromin prepared from rat leprosy bacillus.

From a study of the complement-fixation test in leprosy with the antigens prepared from various acid-fast bacilli it was concluded in a previous publication that complement-fixation tests have not given, and are unlikely to give, any evidence either for or against the genuineness of the cultures of organisms isolated from leprosy lesions. The present study has confirmed this view and has further shown that the absorption of the sera before the tests are performed, does not in any way improve the utility of the tests for this purpose.

REFERENCE

DHARMENDRA and ROSE, R. (1941) *Ind Jour Med Res*, **29**, p. 7

PREPARATION AND USE OF THE WITEBSKY,
KLINGENSTEIN AND KUHN
(W K K) ANTIGEN

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COMPLEMENT-FIXATION of a very high order obtained with this antigen in leprosy and kala-azar has been described (Greval, Lowe and Bose, 1939, Greval, Sen Gupta and Napier, 1939) The antigen was purchased from a Continental source and the question arose almost immediately afterwards whether it could be prepared locally at a reasonable cost To do so was essential before submitting to the profession further recommendations for the use of the complement-fixation in the diagnosis of the aforesaid diseases

THE PREPARATION

According to the original description (Witebsky, Klingenstein and Kuhn, 1931) the antigen is a solution in benzol of the (i) alcohol insoluble, (ii) pyridin soluble and (iii) acetone insoluble fraction of the human tubercle bacillus In the

details which follow the writers have adhered as much as possible to the original description

1 *The cultures of the bacillus*—These were obtained from the laboratory of the Tuberculosis Inquiry of the Indian Research Fund Association working under Dr A C Ukil, at the All-India Institute of Hygiene and Public Health, Calcutta. Some cultures were taken after they had served their purpose in diagnosis, others were especially grown. The medium used was glycerine agar. At least 12 cultures from half as many cases were made, allowed to grow for 2 months, scraped and used for one lot of the antigen. For the purpose of the rest of the description the details of the preparation of the last lot are given.

2 *The bacterial mass*—From 12 tubes and 1 surface of Roux flask the growth was scraped, suspended in 50 c c of saline and autoclaved for half an hour. The killed bacilli were collected by filtration and dried in a desiccator for 2 days (constant weights not necessary). The weight was 1.078 grammes.

3 *Extraction with alcohol*—Twenty-seven c c of alcohol (20 times by weight) were added to the dried mass which was extracted with a reflux condenser over a water-bath for 3 hours. The bacilli were again collected by filtration and dried in an incubator. The weight of the dried mass was 0.788 gramme. *This was the alcohol insoluble fraction.*

4 *Extraction with pyridin*—The dried mass was extracted in a 50 c c Soxhlet apparatus with pyridin for 5 hours over a glycerine-bath at 130°C. From the pyridinic solution the solvent was removed by distillation over a sand-bath. The weight of the dried residue was 0.1652 gramme. *This was the alcohol insoluble and pyridin soluble fraction.*

5 *Extraction with acetone*—The residue was extracted with 20 c c of acetone with a reflux condenser over a water-bath for 3 hours. The acetonic solution was filtered off. The residue on the filter was washed with 3 c c fresh acetone and dried in an incubator. The weight of the dried mass was 0.075 gramme. *This was the alcohol insoluble, pyridin soluble and acetone insoluble fraction.*

6 *Solution in benzol*—The residue was dissolved in 7.5 c c of benzol in a small pestle and mortar (total quantity made up after evaporation due to exposure) to give a 1 per cent solution. The solution, unlike the commercial preparation, was milky. Fifteen c c of benzol were added by instalments of 5 c c to obtain a clear solution. *The further addition of benzol is a departure from the original technique.*

7 *Addition of lecithin*—To two parts of the benzolic solution was added residue from one part of a 1 per cent alcoholic solution of lecithin. *This was the desired antigen.*

The anticomplementary titre, the reaction with pooled kala-azar serum and the reaction with a batch of 25 Wassermann positive sera, of the locally prepared antigen and the commercial antigen, were the same.

The hæmolytic activity of the antigen was determined by leaving three volumes of its selected dilution with one volume of the r b c suspension for half an hour.

at 37°C Lysis did not occur. Traces of lysis were excluded by leaving the tubes in the cold overnight. Incidentally this determination, though carried out for each lot of the antigen, was not mentioned in the communications on leprosy and kala-azar referred to above.

The special feature of the writers' technique of fixing complement with the W K K antigen is that the dose used is the maximal non-anticomplementary quantity of the antigen. Further, this quantity is non-hæmolytic even when multiplied by three. Possible variations in the concentration of the benzolic solution do not interfere with the determination of the dose. This dose fixes a constant quantity of the complement, measured in m h d with a constant serum.

Incidentally, the special antigenic constituent of the local tubercle bacillus must be greater than that of the bacillus from which the commercial preparation was made.

The locally prepared antigen, like the commercial antigen, keeps well when stored in a cool and dark place. If stored in a refrigerator it is taken out a day before the test and left at room temperature overnight. If not perfectly clear next day, it is left for half an hour in an incubator at 37°C.

The quantity of the benzolic solution needed for a day's work, confirmation of anticomplementary titre and 12 to 24 tests, need not exceed 0.2 c.c.

THE TEST

The technique of the test has been described previously (*vide supra*). One correction and two additions may be made now. (i) In Volume 27 of this *Journal* the figure on page 185, para 2, line 2 should be 137 instead of 132. (ii) The writers now undertake the test, which is linked to the Wassermann reaction in their laboratory, on a day when the complement for the latter reaction is of optimal reaction and titre (Greval, Chandra and Das, 1940). The latter reaction is undertaken on 5 days a week and the complement is stored for 24 hours if it is not convenient to work on kala-azar or leprosy on a particular day. (iii) In determining the anticomplementary titre of the antigen a crystal-clear tube is not considered necessary.

ACKNOWLEDGMENTS

Thanks are due to Dr A C Ukil of the All-India Institute of Hygiene and Public Health, Calcutta, for supplying cultures of human tubercle bacillus and to Dr S Ghosh of the School of Tropical Medicine, Calcutta, for aid in translating the original description of the makers of the antigen.

SUMMARY

1. An antigen from the human tubercle bacillus, fixing complement with sera from cases of leprosy and kala-azar, like the W K K antigen, has been prepared.

2 In the complement-fixation test for leprosy and kala-azar previously described, complement of optimum reaction and titre, as determined for the Wassermann reaction, only is now used

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THE RELIABILITY OF GUINEA-PIG INOCULATION TEST FOR THE DIAGNOSIS OF HUMAN TUBERCULAR AFFECTIONS

BY

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AMONGST the laboratory methods employed for the diagnosis of tubercular affections, animal inoculation holds a very high place. It is said that for the detection of human type of tubercle bacilli guinea-pig inoculation is 'the court of last appeal' (Todd, 1939). Indeed it has been stated that the subcutaneous injection of a single tubercle bacillus may succeed in producing disease (Wa'moschur and Stoecklin, 1927, Doerr and Gold 1932). It is being gradually recognized, however, that the test is not as infallible as it was supposed to be. Recent work has shown that the material such as a specimen of sputum must contain more than 100,000 organisms per c c before they could be detected by ordinary microscopic examination and that guinea-pig inoculation was a thousand times more sensitive a procedure for detecting them. It is believed that as few as 10 to 100 organisms suffice to give a positive result (Nicholson, 1934).

Guinea-pig inoculation was used as a routine procedure in the Central Clinical Laboratory of King Edward VII Memorial Hospital, Bombay, during the course of nearly 4 years, i.e. from 1927 to 1930 inclusive. In Table I are given a few of the clinical notes and the results of guinea-pig inoculation and culture on specimens of material sent to the Laboratory. It would be seen from it that out of 9 specimens sent, guinea-pig inoculation is positive in 1 case, while cultural examination is positive in 3 cases. The 1 case in which the animal inoculation is positive also shows the growth of tubercle bacilli by cultural examination. Advent of refined and selective media for the growth of *M. tuberculosis*, such as those of Corper, Lowenstein, Petragnum and others, has made cultural examination a more sensitive procedure than before and the employment of these media gave so good results in our hands that, after the year 1931, guinea-pig inoculation was practically given up as a routine procedure in our Laboratory.

Guinea-Pig Inoculation Test in Tubercular Affections

TABLE I
Showing the result of guinea-pig inoculation and cultural examination of pathological material sent for detection of tubercular infection

Serial number	Date	Name	Age	Sex	Register number	Nature of material	Culture	Guinea pig inoculation	Short notes on the clinical findings
1	13-8-27	Mr De Abro	35	Male	A/2914	Urine	Negative	Negative	T B lung and stone left kidney —Fever without rigors for one month and a half Prior to this patient was having fever with rigors in the beginning on alternate days and later at the interval of 4 or 5 days for about 15 days Patient had pleurisy with effusion about a year and a half back and passed a stone about 4 years back Patient had marked clubbing of fingers Sputum contained tubercle bacilli on microscopic examination In the wards patient had occasionally a slight rise of temperature in the evening
2	30-8-27	Mr Yusuf Karim		Male	A/8499	Pleural fluid	Positive	Positive	
3	27-3-28	Mrs Hara bai Absbai	21	Female	B/1033	Urine	Negative	Negative	T B spine —Pain epigastrium, left iliac fossa and back for 3 to 4 years Difficult and painful micturition Signs and symptoms of T B spine X ray erosion of tenth dorsal to fifth lumbar bodies of vertebrae Slight rise of temperature in the evening
4		Mrs P K Iyer		Female	339	Urine	Negative	Negative	

C B Dhurandhar

Curious liver—Generalized enlargement of abdomen. Signs of ascites present. Spleen enlarged and hard. Liver enlarged hard but not tender, duration of illness about 3 months. In wards patient was having hectic type of temperature.

T B caecum?—Pain in the right iliac fossa and lumbar region and occasionally in the right testis for 6 months. Prior to this trouble patient had suffered from fever for few days and had mucus occasionally. A tumour of the size of an egg freely movable but tender on palpation could be felt in the right lumbar region. Patient left hospital against medical advice.

Pain in the right lumbar region for 7 days. Patient had hemiplegia of recent origin. Difficult breath sounds, crepitations right lung at the base. Sputum shows few tubercle bacilli on concentration. Occasional rise of temperature in the evening.

11-8-30	Balkrishna man	Lax	14	Male	D/5265	Ascitic fluid	Negative	Negative	Negative	
4-4-30	Mr. Bhimchand		25	Male	D/2230	Urine	Negative	?		
27-12-29	Mr. Sayad Husein	Amir	45	Male	C/8001	Sputum	Negative	Negative		
						Joint fluid	Positive	Negative		
						Pus from P M material	Positive	Negative		

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In the year 1935 when this question was being reviewed, it was decided to carry out a few experiments with animal inoculation before finally giving it up as a routine procedure in the Laboratory

METHOD OF OBTAINING CULTURES

With this aim in view strains of tubercle bacilli were isolated from specimens of sputa from cases of pulmonary tuberculosis on Lowenstein's media. The procedure employed was as follows. 0.5 c.c. to 1.0 c.c. quantity of sputum collected in a sterile Petri dish was washed in saline and transferred to a sterile 100 c.c. centrifuge tube. To it were added about 3 c.c. to 5 c.c. of 15 per cent sulphuric acid. The sputum was thoroughly mixed with acid by means of a sterile rod and the acid was allowed to act on it for 15 minutes. Then about 30 c.c. of sterile distilled water were added and the tube was centrifuged. Supernatant liquid was thrown away and a fresh quantity of 50 c.c. of distilled water was added and the deposit was mixed with it by means of a long rod. This procedure was repeated once again to get rid of as much acid as possible. The deposit was planted on Lowenstein's media and the culture tubes were sealed with paraffin and incubated at 37°C. Growth appeared within 8 to 10 days and was luxuriant in about 3 weeks' time.

MASSIVE DOSES

A batch of 12 guinea-pigs was inoculated subcutaneously with massive doses of different strains of tubercle bacilli obtained from the first sub-culture after a primary culture. Out of 12 guinea-pigs thus inoculated 9 suffered from infection, while 3 remained negative. These animals were kept under observation generally for a period of 3 months at the end of which, unless they died before, they were sacrificed and a thorough examination was made naked eye, microscopically from smears made out of the material and by histological examination of tissues. Three animals failed to show evidence of tubercular infection after a searching examination carried out in the manner described above. This showed that a certain number of guinea-pigs failed to develop lesions even when massive doses were employed.

Corper (1918), who has done considerable amount of work on this question, has shown that injection of massive doses of human tubercle bacilli in guinea-pigs produce an infection in 98 per cent of animals inoculated, the actual figures in his experiments being 86 animals positive out of a group of 88 inoculated. The explanation given was that the two negative results were due to low virulence of the strains used.

Not being satisfied with the results obtained it was decided to try massive doses in another set of 14 guinea-pigs. The procedure employed was the same as in the previous experiment.

The animals used were approximately of the same age and weight except 2 (440 g. and 568 g.), i.e. 12 of the animals used were of a weight varying between 300 g. and 400 g.

TABLE II

Serial number of guinea pigs	Strain number	Number of weeks the animal lived	How died	Severity of lesions	WEIGHTS OF THE ANIMAL IN G	
					Initial	Final
1	S 32	25	K	++	440	510
2	S 39	3	D	+++	317	294
3	S 37	14	K	+++	325	353
4	Blood	3	D	++++	310	270
5	S 36	14	K	++	350	391
6	Pus cold abscess	13	K	+++	330	443
7	Pleural fluid	14	K	+	350	523
8	S 40	14	K	++++	565	598
9	S 42	5	D	+++	365	380
10	S 46	14	K	+++	310	480
11	S 44	17	K	+	335	512
12	S 50	17	K	++++	365	532
13	S 48	13	D	++	318	392
14	S 49	17	K	+	371	536

+ = One or 2 glands enlarged.
 ++ = In addition to the above other organs affected but no caseation.
 +++ = Caseation in other organs
 ++++ = Extensive caseation
 K = Killed
 D = Died

ANATOMICAL AND HISTOLOGICAL CHARACTERS OF THE LESIONS

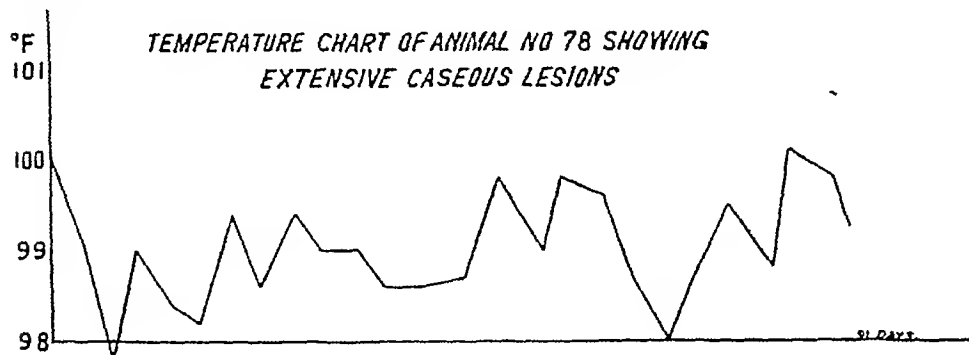
The results obtained in this second experiment with massive doses show that none of the strains employed were absolutely avirulent but they show marked variation as regards their virulence. In Table II animal No 4 died within a period of 3 weeks and an autopsy showed extensive caseation of the viscera, while animal No 14 showed just one enlarged, fibrotic and slightly caseated gland in the groin after the lapse of 17 weeks at the end of which the animal was killed. From the fibrotic nature of the lesion the impression gained was that, had the animal not been sacrificed, the lesion would probably have healed. Its progressive increase in weight by 165 g during the course of 17 weeks also lends support to this view.

According to the anatomical and histological characters, the results obtained fall into four groups—marked in the table +, ++, +++, +++++ Three animals showed enlarged one or two lymph glands which showed slight evidence of caseation and marked fibrosis Another group of 3 animals showed, besides a few enlarged glands, certain amount of cellular infiltration in the viscera Five animals showed enlarged lymph glands and viscera with evidence of caseation in both of them Three animals showed the characteristic picture of extensive caseation in the lymphatic glands, liver, spleen and other viscera These results indicate that when different strains of tubercle bacilli are injected in guinea-pigs they show considerable variation in the type of lesions that they produce in these animals

Weight—One of the signs which gave an indication as to whether an animal inoculated with suspected tuberculous material was developing a lesion or not was its loss of weight Emaciation is a characteristic sign of all tubercular infection In Table II are given the weights of the animals at the beginning of the experiment and their weights taken at the time of their death, or killing them Out of the 14 animals only 2 show a loss in weight, while the remaining 12 have gained in weight In animals in which at autopsy the lesions were of a regressive nature it is not surprising that the weight went up but what is astonishing is the fact that even when the animal (i.e. No 12) showed an extensive caseation of viscera at the time when it was killed after the lapse of 17 weeks, its weight had gone up by 167 g In another animal (No 8) which showed also extensive caseation the rise in weight was 33 g, while the third one (No 4) which showed similar lesions lost its weight by 40 g It is interesting to note that this animal died within a period of 3 weeks, while the other 2, which showed an increase in weight, were killed one after a period of 14 weeks and the other after 17 weeks These findings are in agreement with our previous experience when for the purpose of demonstrating lesions to the class we had noticed on several occasions that a record of weights taken regularly showed that the weight of the animal either remained stationary or actually went up, although on opening it showed marked lesions A record of weight with a view to detect a loss or otherwise, does not appear to be an useful indication that the animal was or was not developing the infection This finding is in agreement with the view expressed by Tulloch, Monroe, Ross and Cummings (1924) who, on the basis of animal experiments with 100 strains of tubercle bacilli isolated from sputum, conclude that average weekly weighing does not give a true index of whether an animal is or is not suffering from tuberculosis

Temperature—Another sign which was supposed to indicate that the animal was developing infection was a rise in temperature Calmette (1923) mentions that an infected animal at the end of 3 weeks constantly showed a temperature above 39.5°C, i.e. 103°F A regular record of the day-to-day rectal temperature of the animals infected with massive doses was kept during the period of observation In none of the animals a rise in temperature above 102°F was noted at any time A record of the temperature of some of the animals was kept for about a fortnight before the animal was used in the experiment with a view to find the normal variation in our animals and it was noted that the normal temperature varied between 97°F to 101.8°F While taking these observations we were struck

with the fact that struggling on the part of the animal, prior to taking the temperature, shot its temperature up by two to three degrees. In spite of this when records in animals injected with massive doses were made none of the animals ever showed a rise as high as 103°F which Calmette mentions as being constantly present. The following Temperature Chart is an example of the type of temperature record obtained in animals which on post-mortem examination showed extensive caseation in the viscera —



Injection of graduated doses — The results outlined above show that there is a considerable variation in the virulence of different strains isolated from human sputa. That probably is the reason why such a marked difference was noticed in the characters of the lesions. In order to find out whether this variation in the virulence could be responsible for the negative results obtained when animal inoculation was employed from the diagnostic point of view the following experiments were carried out. In this series, graduated doses of known number of organisms were injected into guinea-pigs. It has been said that 10 to 100 organisms sufficed to give a positive result. We started, therefore, from 100 and increased the number to 100,000. Table III gives the dosage employed, the number of animals inoculated and the number of positive and negative results —

TABLE III

Number of organisms	Number of animals injected	Infected.	Not infected
100	7	2	5
1,000	34	24	10
5,000	4	3	1
10,000	2	2	<i>Nd</i>
100,000	2	2	<i>Nd</i>

From Table III it would be readily seen that when 5,000 or more organisms were injected out of 8 animals inoculated only 1 failed to develop the infection. In the light of the work on massive doses, referred to above, it is not at all surprising that an occasional animal should fail to get infected when the dose employed was not a large one. When the dose injected was smaller, i.e. 100 organisms in a group of 7 animals and 1,000 organisms in a batch of 34 animals, it is found that out of 41 animals injected, 15 showed a negative result. All the animals were injected with strains which on injection of massive doses showed evidence of infection, so that there was no doubt as regards the pathogenicity of the strains employed. The strains used in these experiments were the same that were employed for the second series of experiments with massive doses. The animals were given the injections subcutaneously and were observed for a period of 12 to 17 weeks and were killed at the end of that period if they did not die before. When an autopsy was performed a thorough naked-eye, bacteriological, cultural and histological examination was made with a view to detect even if traces of infection existed. Fifteen negative results out of 41 when both groups were considered, or 10 out of 34 when the group with 1,000 organisms was considered, show that these doses of organisms were not sufficient to produce an infection in a guinea-pig, if the strain used was not sufficiently virulent. When 1,000 organisms were used, a dose was employed which was ten times the maximum of the dose which is ordinarily said to produce infection in guinea-pig and yet 29.4 per cent, i.e. nearly 30 per cent of the animals in our series, failed to get infected. If both the groups are taken into consideration the percentage works out at 36.6. This would mean that approximately one out of every 3 animals injected with material which contains 1,000 or less organisms would not give a positive result even though the material may contain tubercle bacilli if the virulence of the organisms was low. These findings detract considerably from the value of guinea-pig inoculation test as 'the court of last appeal'.

Cultural examination with small doses—Smith (1933) has given a description of Lowenstein's technique of isolating tubercle bacilli from blood. By following this technique it has been mentioned that tubercle bacilli could be isolated from the blood of cases suffering from different types of tubercular affections. Adopting the same technique, we tried to isolate the *M. tuberculosis* from the blood of persons suffering from pulmonary tuberculosis. After performing 40 blood cultures, when we could not succeed in isolating tubercle bacilli from the blood in florid cases of pulmonary tuberculosis, we decided to carry out a few experiments in order to see whether the technique as carried out by us was in any way detrimental to the growth of the organisms. A sample of citrated blood was obtained and a small number of (about 50) organisms were added to the blood and then the rest of the process of laking of blood and digestion was carried out in the usual manner. On seeding the deposit on Lowenstein's media a number of colonies of tubercle bacilli were grown and although a count of the colonies was not made a large percentage of organisms was left unaffected by the process of digestion as shown by the number of colonies grown on the media. This experiment was repeated on two or three more occasions and in every instance a positive culture was obtained. It might be of interest to add that after a large number of trials we succeeded only once in isolating *M. tuberculosis*.

from the blood of a case of pulmonary affection. That strain was used in the second series of experiments with massive doses and it produced in 3 weeks a fatal infection in the animal which on autopsy showed extensive caseo-necrotic tubercular lesions in the lymph glands and viscera.

DISCUSSION

Feldman and Magith (1931) working on the reliability of guinea-pig inoculation for diagnosing human tuberculous material came to the conclusion, that a subcutaneous or intraperitoneal injection into guinea pigs and performing necropsy on those that were living at the expiration of 8 weeks was a thoroughly dependable procedure. This view is generally held by most workers. The experiments outlined above, however, tend to show that the claims made for the sensitivity of guinea-pig inoculation as a test are exaggerated and that whether the animal would give a positive or negative result would depend principally on three factors, viz (1) the dose of micro organisms contained in the material, (2) the virulence of the tubercle bacillus and (3) the sensitivity of the test animal. Our experiments on graded doses bring out in a clear manner that the number of microbes contained in the inoculum determines to a certain extent as to whether the animal would get infected or not. When the number of organisms in the inoculum was of the order of 100 to 1,000 organisms, there was a 1/3 chance of the animal not getting infected although the material contained pathogenic tubercle bacilli. As already stated above the pathogenicity of the strains used was assured before it was employed for experiments with graded doses.

The question as regards the variation in virulence of tubercle bacilli is a debated one. Workers such as Calmette (*loc cit*), Griffith (1922) and others do not believe in the difference in virulence of freshly isolated strains. While Corper and others hold an opinion that such variation in virulence does exist. Topley and Wilson (1936) came to a conclusion that 'very little exact information, however, based on an adequate number of animal tests is available about the difference in virulence of freshly isolated strains of the same type or about the factors that are responsible for changes in virulence occurring *in vitro* or *in vivo*'. Admitting that the number of experiments performed by us is not large they clearly point to the fact that such a variation does exist in strains which are freshly isolated. If a micro-organism possesses high virulence few microbes inoculated into animal would set up a fulminating infection, but if their virulence is low and the number injected is limited, defensive mechanism of the animal may succeed in overcoming the infection. Thus, we believe, is the reason why in our experiments we got so many negative results when the number of microbes inoculated was small. In considering the result obtained the third factor, namely the resistance of the animal, must also be taken into consideration. Whether the resistance of the local breed of animals is more than what obtains in cold countries, it is difficult to say. It may be pointed out, however, that the weight and the temperature of the animal which give a measure of their fight against the offending germs show in our experiments that the animals put up a good resistance. Some of the animals, which on being killed

after 17 weeks showed extensive caseo-necrotic lesions, did not show any loss of weight or an appreciable rise in temperature. It may be argued that those strains were not sufficiently virulent but the very fact that such marked lesions were produced in the bodies of the animals indicates that they possessed sufficient pathogenic powers. Experiments designed to demonstrate the power of resistance of local breed of animals are under way and until they become available the question of resistance will have to be kept *sub judice*. In the meantime, it could be stated that from the point of view of diagnosis guinea-pig inoculation is not as sensitive a procedure as it is reported to be.

SUMMARY

1 Strains of human tubercle bacilli isolated from cases of pulmonary tuberculosis vary considerably in their virulence

2 Guinea-pigs showing massive caseation in the body do not necessarily lose weight or show a rise in temperature

3 Inoculation of 1,000 organisms or less of different strains failed to produce an infection in 30 to 36 per cent of animals

4 From a diagnostic point of view, guinea-pig inoculation test cannot be considered as 'the court of last appeal'

ACKNOWLEDGMENTS

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PREPARATION OF PEPTONE FOR BACTERIOLOGICAL WORK

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THE war has created a need for manufacturing locally commodities which were hitherto imported, and bacteriological peptone is one such. The imported peptones being proprietary products, the details of the process of manufacture are not known. So an attempt was made to develop a technique of manufacture of peptone and success has been achieved by papain digestion of pork. A peptone of excellent bacteriological efficiency and at the same time of remarkable cheapness of production has been obtained. For the benefit of laboratories in India interested in obtaining good peptone at a cheap cost the method of preparation is herewith given.

WHAT IS PAPAIN?

Papain is a plant proteolytic enzyme believed to consist of a mixture of proteinase and polypeptidase. The former acts on whole proteins to yield proteoses which are then broken down to peptones and polypeptides. Amino-acid production is very limited although not impossible, and takes place appreciably only when the papain has been activated by hydrocyanic acid or hydrogen sulphide. Papain acts optimally at pH 5 to 7 or more accurately at the iso-electric point of the substrate, and at a temperature of 50°C.

PREPARATION OF PAPAIN

Papain is easily prepared in the following way. The latex of the unripe fruits of *Carica papaya* is collected by scarifying the fruits, preferably, while still on the

tree as in the case of rubber latex collection. The accumulated collections are frozen in ice and brought to the laboratory and treated with roughly three volumes of cold acetone and thoroughly mixed up. When the solid settles down the acetone is poured off and the solid treated twice again in the same way as before with cold acetone and finally spread on a dish and kept against a current of cold air from a table fan. The acetone is thus removed and the material is kept in a vacuum desiccator overnight. Next day the material is well powdered and sieved through muslin. The fine pearl-white powder thus obtained is usually found to be a very active preparation. The fruit, if aseptically scarified, heals up admirably. The acetone after being used in the process can be re-distilled and re-employed. It is possible to recover about 80 per cent of the acetone in a pure state by re-distillation and this cheapens cost.

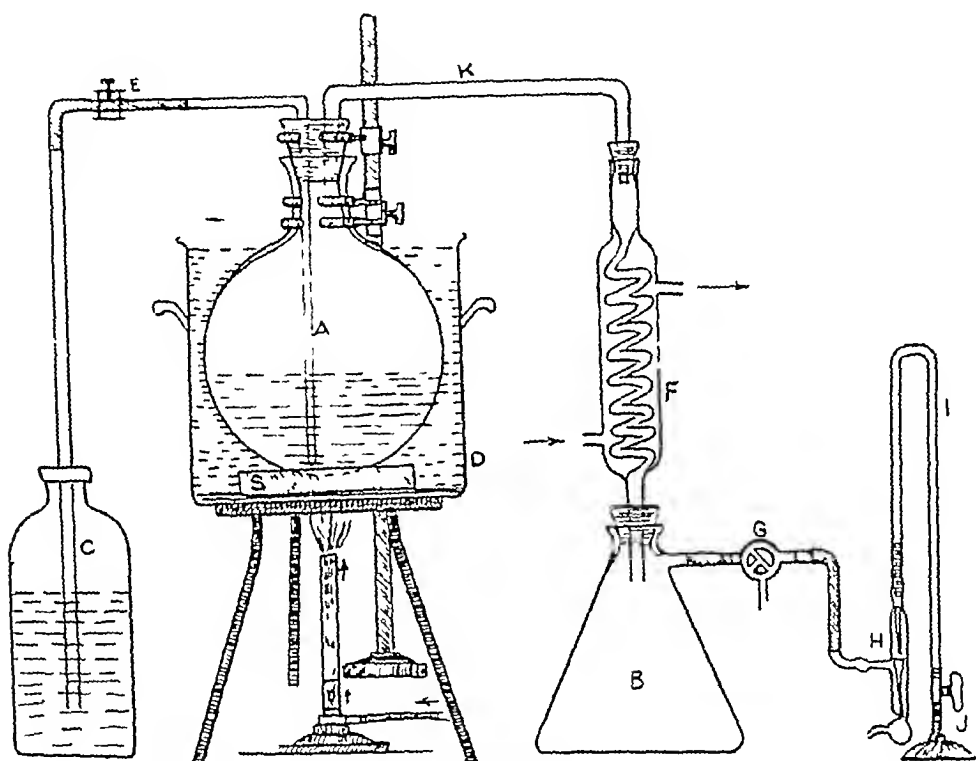
WHAT IS PEPTONE ?

The term peptone in chemistry refers to a protein disintegration product more complex than a polypeptide but not so complex as a proteose. The commercial 'peptones' used in bacteriology are really mixtures of metaproteins, proteoses, peptones, polypeptides and possibly some free amino acids, in which there is relatively a high proportion of peptones and polypeptides. In the disintegration of protein by enzymes a succession of hydrolyses takes place with the formation of a mixture of all the above-mentioned products. The relative proportion of each one of the products varies with the conditions of hydrolysis as well as the specificity of the enzyme. Papain digestion of pork under the conditions described below yields a peptone useful for bacteriological work.

PREPARATION OF PEPTONE

For a small scale preparation of peptone, 6 lb of pork, freed from adhesive lumps of fat and bones, are passed through a fine mincer a number of times. About 2.6 kg to 2.9 kg of minced pork are obtained to which is added an equal number of litres of water and stirred up in an enamelled vessel fitted with a lid. 1/60 to 1/20 by weight of papain (depending on the quality of the papain) and calculated on the weight of the minced pork are ground up to a pulp, diluted to about 200 c.c. with water and then added to the minced pork suspension and well mixed up. The pH of the medium is nearly 5, and does not usually vary outside the range of 5 to 7 and so no adjustments need be made. The digestion of the pork is visible almost immediately the enzyme is added and it is allowed to take place at 50°C by keeping the vessel in a water-bath. A little chloroform (10 c.c.) and a little toluene (10 c.c.) are added which together with the temperature of 50°C protect the pork from bacterial contamination. The digestion is allowed to take place for a whole day (10 a.m. to 4 p.m.). In the evening the almost completely digested material is transferred to well-cleaned glass-bottles and after the further addition of a few c.c. of preservatives the bottles are shaken and kept in the incubator overnight. Next morning a formol titre is done and a further quantity of papain, usually about half the quantity previously used, is added and the digestion allowed to take place at

50°C for another whole day. If subsequent formal titres show only very slight increase in the amino nitrogen the digestion may be assumed to have reached the ultimate state which unactivated papain is capable of. By adopting this procedure one can ensure to a large extent the same quality of peptone being produced in each batch. The product is now filtered through fluted filter-paper. The filtration is conducted inside a refrigerator, as it is time-consuming and exposure of the material



DESCRIPTION OF THE APPARATUS

- | | | | |
|---|---|------|--------------------------------------|
| A | Flask, 5 litre capacity | F | Condenser |
| B | Filter flask 3 litre capacity | G | 3 way tap |
| C | Solution for concentration set up for re charging | H | Filter pump |
| D | Water bath | I, J | Water tap |
| E | Screw clip | K | Glass tube, $\frac{3}{8}$ " diameter |
| | | S | Cork ring |

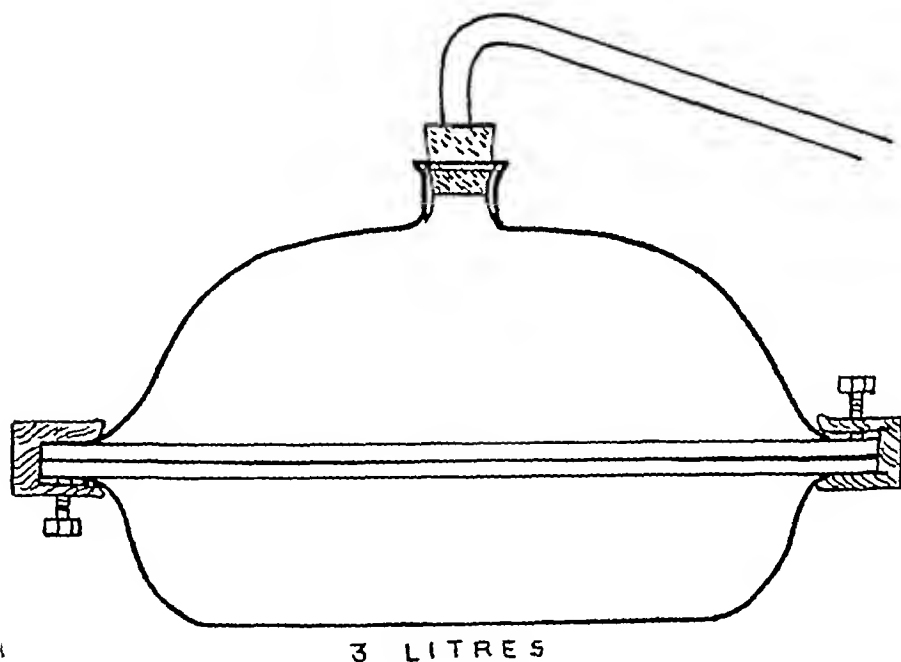
TEXT FIGURE 1

Vacuum distillation apparatus 1

to the atmosphere may contaminate it. The residue on the paper which is composed of mostly fat and proteins of the connective tissue, is washed once with water, filtered and the filtrate added to the original digest. The digest at this stage is at a pH of 5. It is concentrated to a syrup *in vacuo* between 50°C and 60°C with the help of apparatus I (see Text-figure 1) which can be improvised in the laboratory.

The apparatus shown above needs no description. It can at a time cope with 3 litres of the digest. With a pressure head of 50 ft. of water in the tap and a temperature of 55°C about 150 c c of water are distilled off in 1 hour. Towards the final stages of concentration the rate is slower. When a syrupy consistency is reached, distillation becomes difficult due to frothing. At this stage the syrup is transferred to a sterile glass-bottle and kept in the refrigerator. Portions of this syrup about 100 c c are mixed up thoroughly with 100 c c of rectified spirit and put into a drying plant similar to apparatus 1. The alcohol helps the desiccation of the syrup and also prevents frothing. The temperature is slowly raised from 50°C to 65°C and at intervals the semi-dry material in the flask is raked up and moistened with rectified spirit and the drying again continued. In about 6 hours the mass is quite dry. After keeping in the vacuum desiccator overnight the peptone can be taken out in lumps. These are ground up and sieved through muslin cloth to obtain a fine powder.

For the final drying, instead of the small flask an evaporating pan fitted with a detachable lid as in Text-figure 2 is under construction. This would minimize time and labour, as well as the amount of alcohol used up.



TEXT-FIGURE 2

Evaporation pan to take the place of flask 'A' Apparatus 1
in the final drying

As regards the yield, from 6 lb. of pork costing Rs 3 and 60 g papain costing Re 1-8 and rectified spirit costing Re 1 (duty free) about 400 g of peptone can be obtained.

Several samples of peptone prepared by the above method have been thoroughly tested in our laboratories and found to compare favourably with foreign peptones in all kinds of bacteriological work. A sample was also sent to the Central Research Institute, Kasauli, where it was tested by the kindness of the Director. When used in a variety of media the peptone was reported to give good growth of all organisms tested, and a peptone water made from it used as a medium for the Rideal-Walker test gave a figure similar to that of the standard medium laid down for the test. It was also reported to give a strong cholera-red reaction and good indol production with *V. cholerae* and to give good Voges-Proskauer and methyl-red reactions with suitable organisms. The opinion was expressed that the peptone was suitable for general bacteriological purposes in substitution of many commercial brands.

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AN IMPORTANT ANTIGENIC DIFFERENCE BETWEEN HÆMAGGLUTINOGENS M AND N

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THAT it is easier to produce an anti-ON serum and later by absorption an anti-N fluid, than an anti-OM serum and an anti-M fluid is the writers' experience (Greval, Chandra and Woodhead, 1939) That the anti-ON serum can be exhausted of anti-N substance by repeated absorption with OM red blood cells, whereas the anti-OM serum cannot be exhausted of anti-M substance by repeated absorption with ON red blood cells is also known (Wiener, 1939) With the first observation (easier preparation of anti-N fluid) some workers are not in agreement (Taylor and Ikin, 1939)

ANOTHER DIFFERENCE NOT PREVIOUSLY REPORTED

In the writers' method of preparing the typing anti-M and anti-N fluids the unabsorbed antisera are first titrated with OM and ON red blood cells, to determine their anti-O potency and specificity for M and N The anti-OM sera have been found to be distinctly weaker in this preliminary titration On the whole their titre is about a quarter of that of the anti-ON sera The difference is not one of quality only but of quantity also The total protein content of anti-ON sera has been found on the whole to be four times that of the anti-OM sera The comparison has been made by the foam test The dilutions of anti-OM and anti-ON sera which

show nearly the same anti-O potency also show the same degree of frothing on shaking, although the latter have been diluted nearly four times as much as the former

Anti-OM sera of high anti-O potency are also found. So far the writers have not been able to prepare anti-M fluids from them. Usually they are either non-specific or falsely specific for ON cells. Absorption with red blood cells of either type makes them inert.

GENETIC SIGNIFICANCE OF THE DIFFERENCES

The response to N in the production of an antibody is of a higher order than the one to M. The antibody produced is appreciably greater. Further, the antibody produced does not react with N only. It is also absorbed and exhausted by M. Evidently, N is not only more effective but also possessed of more potentialities as an antigen. A hypothesis is advanced that the phenomenon is a proof of the greater age of N if zoogenesis be the process of evolution. N appeared earlier than M which came into being as a result of disappearance of certain potentialities from the former. Predomination of N in the Australian aborigines (Birdsell and Boyd, 1940) points in the same direction. Further, in this hypothesis, the characters M and N cannot be independent and indivisible biochemical entities like the characters A and B.

OBVIOUS FALLACIES IN WORK WITH IMMUNE ANTI-A AND ANTI-B SERA, PRIOR TO THE KNOWLEDGE OF M AND N, AND A SIMILAR DANGER FROM OTHER HÆMAGGLUTINOGENS

Prior to the recognition of the hæmagglutinogens M and N some workers prepared antisera against A and B by immunizing rabbits against red blood cells A and B, irrespective of the M and N content of the cells (Snyder, 1929). It is obvious that the hæmagglutination was brought about not only by anti-A or anti-B bodies but also by anti-M and anti-N bodies. Anti-N body being of a higher order in titre is more likely to have interfered than anti-M body.

In the anti-A and anti-B sera now prepared anti-M or anti-N body is removed by absorption (Boyd and Boyd, 1937). anti-AM serum is absorbed with BM red blood cells and anti-BM serum with AM red blood cells. Similarly, anti-AN serum is absorbed with BN cells and anti-BN serum with AN cells.

While the danger from interference by M and N is over the one from interference by P, Q, X, E and e (Wiener, *loc cit*) remains. These hæmagglutinogens appear to exist and very little is known about their potentialities in the production of immune antibodies. Artificially produced immune antibodies against A and B, therefore, must be considered less reliable than the naturally occurring iso-antibodies, iso-hæmagglutinins ('isonins' of the writers—Greval, Chandra and Woodhead, 1941) a and b. The observation is of special interest in determining the blood group from stains for medico-legal purposes.

In the determination of the type, when antifuuids prepared from artificially produced immune bodies must be used, testing with three antifuuids is recommended.

(Taylor and Ikin, *loc cit*) This recommendation is indispensable in medico-legal work in spite of the fact that data on the easily determinable heredity of M and N are now sufficiently large. The three antifuuids must, of course, be prepared from the red blood cells of three subjects.

ANTI-N BODY IN BLOOD TRANSFUSION

In view of the high order in titre of the anti-N body the possibility of its formation in the system of a recipient of M type, after transfusion with blood of N type, must not be ignored. A second transfusion of blood of N type may kill. Direct matching of bloods of the donor and the recipient, therefore, is doubly important in second or subsequent transfusions.

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RICE DIETS AND BERIBERI.

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IN *Indian Medical Research Memoir* No 32 (Aykroyd, Krishnan, Passmore and Sundararajan, 1940) the relation between rice and beriberi in India was discussed at length. It was pointed out that in the Madras Presidency beriberi as a serious public health problem is largely confined to the north-east coast area, including the Ganjam, Vizagapatam, Godavari, Kistna, Guntur and Nellore districts (Northern Circars). In this area the poorer classes, i.e. the great majority of the population, prefer raw to parboiled rice, whereas in most other rice-eating areas in Madras and elsewhere in India the reverse is the case. Parboiled rice retains the greater part of the vitamin B₁ originally present even after a high degree of milling, because in the process of steaming the vitamin diffuses into the endosperm, raw rice, on the other hand, loses most of its vitamin B₁ on decortication. About 70 per cent of the rice-eating population of Madras consumes machine-milled rice and this practice is as common outside the beriberi area as inside it. The relative freedom of most of South India from beriberi is due to the fact that parboiling mitigates losses of vitamin B₁ in the milling process, and also to the fact that the vitamin is less easily removed by washing from parboiled than from raw rice (Yang, 1938, Swaminathan, 1941).

SURVEYS

The present paper describes the results of diet surveys carried out in the village of Alamuru in the Godavari delta. The only cereal grown in this area is rice. The rice mill is a prominent feature of the landscape. Driving through the district one observes large mills at intervals of 10 miles or thereabouts, and even in comparatively remote villages small mills serving the village itself and perhaps a few neighbouring ones are to be found. Beriberi is common in the Godavari district

In January 1940 the diet of 34 village families was studied for a period of 20 days. In September of the same year a further survey of 16 families, 10 of which were included in the first investigation, was carried out. This survey lasted 10 days. The usual methods of the Laboratories (Aykroyd and Krishnan, 1937) were followed. Average intake of various foods at the two periods of survey is shown in Table I —

TABLE I

Intake of various foods (oz per consumption unit per day)

	JANUARY	SEPTEMBER
	34 families	16 families
Rice	21.5	21.0
Pulses	0.7	0.8
Leafy vegetables	0.1	0.4
Non leafy vegetables	3.3	2.9
Oils and fats	0.9	1.2
Whole milk	0.6	0.2
Butter milk and curds	9.0	5.0
Fruits	0.2	0.2
Fish, meat and eggs	0.4	0.1
Jaggery	0.2	0.1
Calories	2,600	2,700

The diet in its general composition resembles that of the poor rice-eater in other parts of India (Aykroyd *et al*, 1940). Ingredients other than rice are present in about the usual proportions. Pulse intake is perhaps a little on the low side, but an equally low consumption has been recorded in many rice-eating groups (Aykroyd, 1939). The figures for intake of butter-milk and curds give an exaggerated idea of total milk consumption in terms of whole milk, since much of the butter-milk was highly diluted. In general the vitamin B₁ content of the non-cereal part of the diet does not greatly differ from that of poor rice diets consumed in other districts outside the beriberi area.

Rice—Both parboiled and raw rice were consumed. Taking all families together, the proportion of these two kinds of rice in the diet was as follows during the two survey periods respectively —

	Raw rice	Parboiled rice
January	9.4	12.1
September	20.5	1.4

(Average intake in oz per consumption unit daily)

Rice immediately after harvesting is not considered very suitable for consumption if husked and subsequently pounded or milled in the raw state. It cooks to a pasty consistency and is said to be difficult to digest. If stored in the husk for 2 months or thereabouts it becomes more acceptable in the raw state. Parboiling, on the other hand, renders freshly harvested rice immediately suitable for consumption. At the time of the harvest in November and December stocks of rice may be low and the new crop required for use immediately by the poorer villagers. Accordingly, they use parboiled rice for a month or so. When the paddy has matured they return to raw rice which they prefer.

The change-over from parboiled to raw rice is well illustrated if we take the consumption of the poorer families (i.e. all families excluding Brahmans and Kammas who in general enjoy an economic position above the average level) in the two surveys. The figures are shown in Table II which includes also the intake of the 10 families common to both surveys —

TABLE II

Intake of raw and parboiled rice respectively in January and September

Survey	Group	Parboiled rice	Raw rice
First survey (January)	21 families (excluding Kammas and Brahmans)	19.4	4.8
Second survey (September)	10 families	2.3	22.6
First survey	10 families	19.1	5.5
Second survey	Same 10 families	2.3	22.3

In the Godavari delta and throughout the rice-eating districts in the Northern Circars, it is the general tendency to consume machine-milled grain and this applies both to parboiled and raw rice. A proportion of families included in the surveys

used home-pounded rice, probably because in the area concerned there has been active propaganda in support of home-pounding. Throughout the district in general the majority of the population makes use of the facilities produced by the ubiquitous rice mills, both large and small.

VITAMIN B₁ AND BERIBERI

Cowgill (1934) and Williams and Spies (1938) have shown that there is a relation between the vitamin B₁/calorie ratio of diets and their 'beriberi-producing' effect. The latter suggested that when the vitamin B₁/calorie ratio falls below 0.250 there is danger of beriberi. Aykroyd *et al* (1940) applied the Williams and Spies' criterion to Indian rice diets and reported that this led 'to a result consistent with the fact that beriberi as a serious public health problem is associated with the consumption of raw milled rice and occurs rarely in the parts of the country in which rice is consumed in the parboiled state'. The vitamin B₁/calorie ratio of diets recorded in the present investigation, calculated on the assumption that all the rice was milled, was as given in Table III —

TABLE III

Vitamin B₁/calorie ratio

Survey	All families	Excluding Kammias and Brahmins
First survey	0.28	0.34
Second survey	0.20	0.22

The vitamin B₁/calorie ratio of the diet consumed at the time of the second survey, when raw rice formed the basis of the diet, was below the danger point.

SEASONAL INCIDENCE

We have frequently been informed by physicians in the beriberi area that the disease tends to be more severe and common during the rainy months of August, September and October, and in general more frequent in the second than in the first half of the year. This tendency is illustrated by figures for monthly admissions to the Government Headquarters Hospital, Guntur, during the years 1926 to 1929, given by Mahadevan and Raman (1930), which are as follows —

January	18	July	43
February	21	August	80
March	32	September	61
April	30	October	68
May	32	November	35
June	46	December	24
Total	179	Total	311

A similar tendency is exhibited by admissions to the King George Hospital, Vizagapatam. On the other hand, figures for in and out-patient admissions for beriberi to hospitals and dispensaries in the East Godavari and Kistna districts during the years 1928 to 1938, kindly supplied by the Surgeon-General, Madras, do not show any striking preponderance of cases in the second half of the year. Admissions during the months July to December were about 10 per cent in excess of admissions from January to June. Figures from large hospitals, where a diagnosis of beriberi can be made with a reasonable degree of accuracy, are naturally more reliable than figures from smaller institutions. We do not at present know how far the custom of consuming parboiled rice for a month or so after harvest and of replacing as the year advances a diet which would protect against beriberi by one which is likely to produce the disease is common throughout the Northern Circars. But if the habit is a general one a possible result would be more beriberi in the second half of the year.

SUMMARY

1 Beriberi is common in the Godavari district. A diet survey carried out in this area in January showed that parboiled rice may be consumed for a short period after the harvest in November to January. At the time of a second survey in September raw rice formed the basis of the diet.

2 The vitamin B₁/calorie ratios of the diets consumed in January and September were above and below 0.250 respectively. According to the Williams and Spies' criterion there is danger of beriberi when the ratio falls below 0.250.

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URINARY EXCRETION OF VITAMIN B₆ BY RATS

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A NUMBER of workers have shown that rats develop a typical deficiency disease, commonly known as 'rat pellagra' or 'rat acrodynia', when fed on diets deficient in vitamin B₆ (György, 1934, Chuck, Copping and Edgar, 1935, Copping, 1936), and that crystalline vitamin B₆ cures this condition (Lepkovsky, 1938, György, 1938, Keresztesy and Stevens, 1938, El Sadr, Macrae and Work, 1938, Chick, Macrae and Worden, 1940). In the present investigation the metabolism of vitamin B₆ in rats fed on diets deficient in vitamin B₆ and diets containing the vitamin in sufficient amounts has been studied.

EXPERIMENTAL

Two groups of young rats (6 in each group), with an average body-weight of 46 g, were fed on the basal diet, deficient in vitamin B₆, shown in Table I. Group I received the basal diet alone, while group II received the same diet with a supplement of 10 µg of crystalline vitamin B₆ daily per rat mixed with the food. The animals were kept individually in metabolism cages. The diets were mixed well and made into a thick paste by adding distilled water, to prevent scattering of food. The rats were given weighed amounts of food, in slight excess of their average food consumption. Food residue remaining uneaten was collected daily, and dried in an air oven at 100°C. The dry weight of the residual food was subtracted from the dry weight of the food given to obtain the food intake.

The test diets were given for a period of 6 weeks. The rats were weighed weekly and urine collected daily. To prevent decomposition, 10 c.c. of 1 per cent sulphuric acid and a few drops of toluol were placed in the bottles used for the collection of urine. The funnels and separators of the cages were washed down daily with small quantities of water and the washings added to the respective urine bottles. The urine and washings were daily filtered. The weekly collections of urine from each group were used for the determination of vitamin B₆.

TABLE I
Composition of the basal diet

Ingredients	Parts
Casein, purified	20 0
Sucrose	30 0
Starch	32 0
Salt mixture	5 0
Coco nut oil	10 0
Cod-liver oil	3 0

Ten μg of crystalline vitamin B₆ and 25 μg of riboflavin were given daily to each rat in addition along with food

The analysis of urine, food and tissues for vitamin B₆ urine—The urine and washings collected over a period of one week from each group were adjusted to pH 7, by the addition of barium hydroxide solution. The precipitate of BaSO₄ was removed on the centrifuge. The rest of the procedure was similar to that described by the author for human urine (Swaminathan, 1941). The results are given in Table II —

TABLE II
Intake and excretion of vitamin B₆ by rats (μg per rat daily)
(Figures represent the daily average per rat)

Experimental period, days	GROUP I			GROUP II		
	Intake	Urinary excretion	Balance	Intake	Urinary excretion	Balance
1-7	0.8	2.8	-2.0	9.0	4.2	4.8
8-14	1.0	2.1	-1.1	9.5	3.8	5.7
15-21	0.8	1.9	-1.1	10.0	4.0	6.0
22-28	0.9	1.8	-0.9	9.0	3.0	6.0
29-35	1.0	1.2	-0.2	9.0	4.0	5.0
36-42	1.0	1.2	-0.2	10.0	3.0	7.0
AVERAGE	0.9	1.8	-0.9	9.4	3.7	5.7

Food and tissues—The method used was similar to that previously described by the author for foodstuffs (Swaminathan, 1940) The results obtained are shown in Table III —

TABLE III

Vitamin B₆ content of liver and muscle ($\mu\text{g/g}$)

Tissues	Group I	Group II	Normal stock rats fed on whole wheat, milk and vegetables
Liver	6.5	11.0	12.0
Muscle	4.1	5.8	5.6

RESULTS

Intake and excretion of vitamin B₆—Table II shows that the rats in groups I and II, ingesting 0.9 μg and 9.4 μg of vitamin B₆ respectively daily, excreted on the average 1.8 μg and 3.9 μg in urine respectively. The quantities excreted per rat were thus 0.9 μg above the intake in group I and 5.7 μg below the intake in group II.

Vitamin B₆ content of the liver and muscle—It will be seen from Table III that there was no appreciable difference in the vitamin B₆ content of the liver and muscle of the rats in group II fed on the basal diet supplemented with vitamin B₆ and that of the same tissues in young stock rats. On the other hand, the vitamin B₆ content of the liver and muscle of the animals in group I, fed on the B₆-deficient basal diet, was lower than in the other groups. This observation, taken in conjunction with the fact that excretion exceeded intake in group I, suggests that the excess vitamin B₆ excreted was derived from the liver and muscle, and possibly from other tissues.

The average urinary excretion of vitamin B₆ per rat in group II, receiving 9.4 μg of vitamin B₆ daily, was only 3.9 μg . The fate of the excess of vitamin B₆ not excreted is a problem for further investigation.

The addition of 10 μg of vitamin B₆ to the basal diet produced a small but definite gain in body-weight. The average weekly increase in weights was 2.3 g and 4.7 g in groups I and II respectively.

Recently, Scudi, Koonas and Keresztesy (1940) have studied the urinary excretion of vitamin B₆ in rats, and have reported that at a high level of dosage (10 mg per kg and above) 50 to 70 per cent of the vitamin was excreted by both normal and deficient rats. At low levels (2 mg per kg) their data were only qualitative.

SUMMARY

1 Balance experiments were carried out on two groups of rats for a period of 6 weeks. Group I was given a basal diet supplying about 0.9 μ g of vitamin B₆ daily per animal. Group II received the same diet supplemented with 10 μ g of vitamin B₆ daily per rat. In the group fed on the unsupplemented diet, the daily excretion of vitamin B₆ per rat was about 1.0 μ g in excess of intake.

2 The rats in group II, each receiving 10 μ g of vitamin B₆ daily, excreted 3.7 μ g of the vitamin, e.g. about 40 per cent of the intake.

3 The vitamin B₆ content of the liver and muscle of the rats fed on the unsupplemented diet was lower than that of the same tissues in the control and the stock rats. The excess of vitamin B₆ excreted by the former may have been derived mainly from the liver and muscle and possibly from other tissues.

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A METHOD FOR THE ESTIMATION OF VITAMIN B₆ IN URINE

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IN a previous communication from these Laboratories (Swaminathan, 1940) a method for the estimation of vitamin B₆ in foodstuffs and animal tissues was described. The method with slight modifications has now been applied to urine, with the object of determining excretion under various conditions. The present paper describes the method, and some preliminary determinations on urinary excretion in human beings, before and after test doses of vitamin B₆, are also included.

EXPERIMENTAL

The various reagents required and the colorimetric procedure are essentially the same as those described before (Swaminathan, 1940). The method includes the following steps —

- (1) Removal of protein derivatives, colouring matter, etc with basic lead acetate
- (2) Adsorption of the vitamin on Fuller's earth (B D H) at pH 3 to 4
- (3) Elution of the vitamin with hot barium hydroxide
- (4) Removal of purine, pyrimidine and imidazole bases with silver hydroxide and the excess of silver as the chloride
- (5) Re-adsorption of the vitamin on Fuller's earth (B D H)
- (6) Elution of the vitamin with hot barium hydroxide and the excess of barium as the sulphate

- (7) Evaporation to a small bulk on the water-bath, treatment in the hot with nitrous acid and making up to a known volume after adjusting pH to 7.4
- (8) Colorimetric estimation of the vitamin B₆ present in aliquots using the diazo reaction

Procedure —A convenient volume of urine (equivalent to a normal 3-hour specimen and containing 50 μ g to 100 μ g of vitamin B₆) was diluted to 1 litre with water. In the case of urines collected after test doses, one-tenth of the volume of the 3-hour specimen was generally used. One hundred ml of N/5 barium hydroxide were then added followed by 50 ml of N lead acetate solution. The mixture was then well stirred, allowed to stand for 10 minutes and centrifuged. The residue was washed once with 200 ml of water containing 10 ml of lead acetate and 20 ml of N/5 barium hydroxide. The excess of lead and barium present in the centrifugate was precipitated by the addition of 10 ml of 10 N H₂SO₄ and removed on the centrifuge. The clear solution (if the solution was not clear, it was filtered using Whatman No. 1 filter-paper) was adjusted to pH 3 to 4 by the addition of sufficient 10 N sodium hydroxide. Ten g of Fuller's earth (B. D. H.) were then added and the mixture shaken vigorously for 5 minutes. The activated Fuller's earth was separated on the centrifuge and washed once with 200 ml of N/10 acetic acid.

In the next stage, the Fuller's earth was washed down quantitatively from the centrifuge tubes to a beaker, with the help of 150 ml of N/10 barium hydroxide. The mixture was heated with constant stirring in a boiling water-bath for 10 minutes, cooled and centrifuged. The residue was washed once with 100 ml of N/10 barium hydroxide. The combined centrifugate was neutralized to pH 7 by the addition of sulphuric acid and the precipitate of barium sulphate removed on the centrifuge. Four ml of N AgNO₃ were then added to the centrifugate, followed by 15 ml of N/5 Ba(OH)₂. The mixture was stirred well, allowed to stand for 5 minutes and centrifuged. The excess of silver and barium present in the centrifugate was precipitated by the addition of 1 ml each of 5 N HCl and H₂SO₄ and the precipitate removed on the centrifuge. The clear centrifugate was adjusted to pH 3 to 4 by the careful addition of sodium hydroxide and diluted to 600 ml with water. Five g of Fuller's earth (B. D. H.) were then added and the mixture shaken vigorously for 5 minutes. The activated earth was separated on the centrifuge, washed once with 100 ml of N/10 acetic acid and transferred to a beaker, using 100 ml of N/10 Ba(OH)₂. The mixture was then heated with stirring in a boiling water-bath for 10 minutes, cooled and centrifuged. The residue was washed once with 50 ml of N/10 Ba(OH)₂. The combined extracts were neutralized to pH 7 by the addition of H₂SO₄ and the precipitated barium sulphate removed on the centrifuge. The clear centrifugate was evaporated to dryness on a water-bath, after being acidified with 1 ml of N HCl. Five ml each of 10 per cent NaNO₂ solution and glacial acetic acid were then added to the residue in the evaporating dish. The mixture was heated on the water-bath for 5 minutes, cooled, adjusted to pH 7.4, filtered and made up to convenient volume (50 ml to 100 ml). Ten ml aliquots were used for the colorimetric estimation of the vitamin B₆ present by

the procedure described before using diazotized sulphanilic acid (Swaminathan, 1940). A blank estimation was also carried out by omitting the addition of the diazotized sulphanilic reagent allowance being made for the blank values in the usual manner.

Recovery of added vitamin B₆ to urine—Different known amounts of vitamin B₆ were added to known volumes of urine. The recovery was good in all cases, ranging from 76 to 94 per cent. The results obtained are given in Table I.

TABLE I

Recovery of vitamin B₆ added to urines

Experiment number	Urines with and without added vitamin B ₆	Total vitamin B ₆ (μg)	Recovery, per cent
1	Urine 3 hour specimen	64	
	" " + 50 μg vitamin B ₆	108	88
2	Urine 3 hour specimen	52	
	" " " + 100 μg vitamin B ₆	140	88
3	Urine, 3 hour specimen	56	
	" " " + 100 μg vitamin B ₆	150	94
4	Urine, 3 hour specimen	56	
	" " " + 150 μg vitamin B ₆	170	76
5	Urine, 3-hour specimen	56	
	" " " + 200 μg vitamin B ₆	240	88

THE EXCRETION OF VITAMIN B₆ BY HUMAN BEINGS

The present investigation was carried out on 5 healthy laboratory workers, the majority of whom were living on a fairly well-balanced diet based on rice and

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including milk, pulses and vegetables in reasonable quantities. In view of the practical difficulties involved in the collection of 24-hour specimens of urines, the excretion of vitamin B₆ in 3-hour specimens of urine, usually collected from 9-30 to 12-30 after breakfast, was determined. The total amount of the vitamin excreted during 24-hour periods was calculated by multiplying the figures obtained for 3-hour periods by 8. Preliminary experiments, given in Table II, showed that the above procedure was justified. Further, it was found that after the ingestion of test doses of vitamin B₆ varying from 10 mg to 50 mg, the increase in the urinary excretion due to the test dose was practically complete during the first 3 hours. Hence the following procedure was adopted. The subjects consumed their usual morning breakfasts. The urines were collected for 3-hour periods, from 9-30 to 12-30 for the first 2 days. On the third day, a test dose of 50 mg of vitamin B₆ was administered orally at 9-30 a.m. and the urine collected up to 12-30 p.m. The 3-hour specimens of normal urine and one-tenth of the volume of the 3-hour specimens collected after the test doses, were analysed for vitamin B₆. In one experimental subject a longer experiment, with varying test doses, was conducted. The results obtained are given in Table III.

TABLE II

Urinary excretion of vitamin B₆ in 24 hours

Subject	Experimental days	Intake of vitamin B ₆	URINARY EXCRETION IN 24 HOURS (μ G VITAMIN B ₆)			
			1st period 9 30 a.m. to 12-30 p.m.	2nd period 12 30 p.m. to 3 30 p.m.	3rd period 3 30 p.m. to 9 30 a.m.	TOTAL
M S	1	Usual diet (supplying about 3 to 4 mg vitamin B ₆ per day)	56	60	340	456
	2	Do Do	56	64	313	430
	3	Do Do	75	70	405	550
	4	Do + 5 mg crystalline vitamin B ₆ at 9 30 a.m.	460	75	345	880
	5	Do + 10 mg crystalline vitamin B ₆ at 9 30 a.m.	600	80	370	1,050

TABLE III

Excretion of vitamin B₆ before and after test doses

Name	Experimental days	Test dose of vitamin B ₆ (mg)	URINARY EXCRETION OF VITAMIN B ₆ (μg)	
			In 3 hours	In 24 hours (3 hours × 8)
M S	1		70	560
	2		62	496
	3		56	448
	4	10	594	
	5	10	625	
	6	10	600	
	7	10	560	
	8	10	560	
	9	50	2 500	
	10		60	480
	11		54	432
R M	1		50	400
	2	50	2 000	
H S	1		60	480
	2	50	2 500	
M	1		53	424
	2	50	1 900	
R	1		65	520
	2	50	2 860	

RESULTS

The urinary excretion of vitamin B₆ in 5 apparently healthy adult males ranged from 400 μg to 560 μg per 24 hours. The diets consumed by these persons contained approximately 3.5 mg to 5 mg vitamin B₆, when calculated according to the figures published before by the author (Swaminathan, 1940). Hence it appears that about 10 per cent of the daily intake is excreted in the urine. In this respect vitamin B₆ appears to resemble vitamin B₁ and nicotinic acid (Swaminathan,

1939, Aykroyd, Krishnan, Passmore and Sundararajan, 1940) After a test dose excretion was increased, about 5 per cent of the test dose being excreted in the first 3 hours after administration. It thus appears that vitamin B₆ is rapidly adsorbed and readily excreted in urine, the increase in the urinary excretion after a test dose occurring mostly in the first 3-hour period. Recently, Scudi, Unna and Antopol (1940) and Spies, Ladish and Bean (1940), using a different method, have reported that about 8 per cent of a test dose of 50 mg of crystalline vitamin B₆ was excreted within the first hour, when the vitamin was administered intravenously. Scudi *et al* (*loc cit*) also found that about 7 per cent of a test dose of 100 mg was excreted in the urine during the first 4-hour period, when the vitamin was administered orally. The results reported in this paper correspond fairly well with those reported by these workers.

DISCUSSION

The method described in this paper should prove to be of value in the study of the metabolism of vitamin B₆ in human beings and experimental animals. The method is highly sensitive. With practice, one to two estimations can be carried out in a day by a single worker.

SUMMARY

1 A colorimetric method for the estimation of vitamin B₆ in urine has been described.

2 An investigation of the urinary excretion of vitamin B₆ in 5 laboratory workers has been carried out. The short interval between the oral administration of the vitamin and its appearance in urine indicates that it is rapidly adsorbed and readily excreted.

3 The normal daily urinary excretion (24 hours) of the vitamin in 5 laboratory workers ranged from 400 to 560 micrograms.

4 Urinary excretion in the above persons, during a period of 3 hours after breakfast, before and after a test dose of 50 mg, was on the average 58 and 2,350 micrograms respectively, about 5 per cent of the test dose being excreted.

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THE VITAMIN B₁ CONTENT OF HUMAN MILK

BY

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MACY, OUTHOUSE, GRAHAM AND LONG (1927) investigated the vitamin B₁ content of human milk by the rat-growth method. They found that 25 ml to 35 ml of the pooled milk from a group of American women were required daily to produce an increase in weight of 15 grammes per week. Williams and Spies (1938) interpreted these results to mean a vitamin B₁ content of 15 to 20 micrograms of vitamin B₁ per 100 ml of milk. Neuweiler (1938), using the thiochrome method of assay and working in Switzerland, reported that the vitamin B₁ content of human milk varied from 5 μ g to 13 μ g. Widenbauer and Heckler (1939) gave an average of 10 μ g per 100 ml for 42 cases (Danzig), with a range from 2 μ g to 36 μ g.

Donelson and Macy (1934) did not observe an increase in vitamin B₁ in human milk when yeast was given to the donors. Similarly, Widenbauer and Heckler (*loc cit*) were unable to influence vitamin B₁ content by giving synthetic vitamin B₁. Morgan and Haynes (1939), on the other hand, reported that when vitamin B₁ content was low it was possible to raise it by administration of the vitamin. They could not, however, raise it beyond 25 μ g per 100 ml.

In the present investigation the vitamin B₁ content of the milk of Indian women, some of whom were suffering from beriberi, or had infants suffering from beriberi, has been investigated by the thiochrome method. Simultaneously, determinations of riboflavin, calcium, ether extractives, lactose and protein present in the milk have been made.

METHODS

Vitamin B₁ in milk is not all present in a free form. Houston and Kon (1939) and Houston, Kon and Thompson (1939) have shown that vitamin B₁ exists both in free and in combined forms in cow's milk and that it is necessary to digest the milk with pepsin and taka-diastase (containing taka-phosphatase) before estimation. This method has been successfully used by Houston, Kon and Thompson (1940) in studying the effect of pasteurization and sterilization on the vitamin B₁ content of cow's milk.

The following method was adopted in the present investigation. 30 ml of milk were acidified with conc HCl to pH 2.5, and 100 mg of pepsin added. The

mixture was kept in an incubator at 37°C for 24 hours. The pH was brought to 4.0 with 30 per cent NaOH, 100 mg of taka-diastase added, and digestion continued at 37°C for a further 5 hours. Three ml of 25 per cent trichloroacetic acid were then added and the mixture well shaken and filtered through dry filter-paper (Whatman No. 44). A portion of the filtrate was kept aside for estimation of riboflavin. Aliquot quantities, usually 5.5 ml, were taken in four glass-stoppered tubes (50 ml capacity) marked 0, 1, 2 and 3, and oxidized with 5 per cent potassium ferricyanide in concentrations of 0.3 ml and 0.5 ml. Thiochrome content was estimated by the method previously described (Aykroyd, Krishnan, Passmore and Sundararajan, 1940). To tube 3, 2.5 µg of pure crystalline vitamin B₁ were added to find out the recovery of added vitamin. The average recovery was found to be 90 per cent and all values have been corrected on the assumption that the observed value is 90 per cent of the actual.

Riboflavin—The protein-free filtrate used for vitamin B₁ estimations was neutralized to pH 7 with 30 per cent NaOH and used for riboflavin estimation. Flavin has the highest fluorescence in ultra-violet light at neutral pH. Hence a phosphate buffer at pH 7 was used for dilutions and for the blank. The flavin was estimated by the method of Whitnah, Kumerth and Kramer (1937) in a fluorimeter.

Calcium—Five ml of milk were ashed in a platinum dish and calcium estimated as oxalate by titration against 0.01 N KMnO₄.

Ether extractives (fat)—The milk was well shaken and 5 ml spread over a filter-paper and dried. This was rolled, put into a paper-thimble and the fat estimated in a Soxhlet apparatus using sulphuric ether as solvent.

Lactose was estimated by titrating the protein-free filtrate against Benedict's quantitative solution (Harrison, 1937).

Protein was estimated by the Biuret method using 0.4 ml of milk (Harrison, *loc cit*). The standard of comparison was a solution of casein from milk.

TESTS ON SUBJECTS WITHOUT BERIBERI

Samples of milk were obtained from mothers attending the Lawley Hospital and the Municipal Child Welfare Centre, Coonoor. These may be divided into two groups, the first consisting of women with infants less than 4 months and the second of women with infants over this age. Nearly all were obtained in the morning by manual expression. A few were obtained by breast pump. The subjects were all poor women, the majority of whom were living on a diet consisting mainly of parboiled milled rice. Parboiled rice retains vitamin B₁ after milling (Aykroyd *et al*, *loc cit*) and is usually preventive of beriberi. A few consumed the millet ragi (*Eleusine coracana*). Apart from the staple cereal, the diets of the subjects contained pulses and vegetables in very small amounts. Their diet was in general what is known in the Laboratories as the 'poor rice-eaters' diet' (Aykroyd *et al*, *loc cit*).

The results of analysis are given in Table I—

TABLE I
Content of breast milk in vitamin B₁ and other constituents
(Subjects without beriberi)

	Age of mother in years	Age of child	Vitamin B ₁ in µg per 100 ml	Thiamin in µg per 100 ml	Calcium in mg. per 100 ml	Other extractives g. per 100 ml	Lactose g. per 100 ml	Protein g. per 100 ml
GROUP 1 INFANTS UNDER 4 MONTHS								
Number of cases and samples	23	23	20	21	17	21	15	
Range	18 to 38	4 to 120 days	5.8 to 20.2	10.6 to 80.0	15.2 to 44.0	1.1 to 5.9	3.2 to 9.5	1.4 to 3.4
AVERAGE	27.0	52.0 days	16.3	58.0	30.1	3.6	7.2	2.1
GROUP 2 INFANTS OVER 4 MONTHS								
Number of cases and samples	25	25	21	23	20	24	19	20
Range	19 to 35	4½ to 11 months	0.2 to 31.0	12.6 to 100	10.0 to 52.0	1.0 to 6.9	4.4 to 9.0	0.5 to 3.6
AVERAGE	24.3	8.3 months	10.5	47.6	27.6	3.0	7.3	2.0

In the case of group 1 (Table I) the vitamin B₁ content of the milk samples ranged from 5.8 μg to 26.2 μg per 100 ml with an average of 16.3 μg . For group 2 the corresponding figures were 6.2 μg to 31.0 μg , with an average of 19.5 μg . According to Williams and Spies (*loc cit*) the ratio micrograms of vitamin B₁ to non-fat calories in the diet should be above 0.30, if danger of beriberi is to be avoided. In the case of human milk forming the sole food of an infant, this means a vitamin B₁ content of 11.2 μg per 100 ml or over. Only two samples were below the level of 11.0 μg . The infants in both cases were under a month old.

The flavin content of the milks varied from 19.6 μg to 80.0 μg per 100 ml in group 1 and from 12.6 μg to 100 μg in group 2. Average values were 58.0 μg and 47.6 μg per 100 ml respectively. Values within the usual ranges were found for the other constituents investigated.

CASES OF MATERNAL AND INFANTILE BERIBERI

The vitamin B₁ content of the milk of a number of mothers showing signs of mild beriberi, some of whom had infants suffering from the disease, was determined. These cases occurred in Guntur and Vizagapatam in the beriberi area, where raw rice is preferred to parboiled rice by the majority of the rice-eating population (Aykroyd *et al*, *loc cit*). The mothers consumed the same type of diet as those in Coonoor, except that raw milled rice was taken instead of parboiled milled rice. Samples 1 to 9 were obtained from St. Joseph's Hospital, Guntur, and the remaining samples from the King George Hospital, Vizagapatam. The same method of assay was employed. The milks were brought to Coonoor for test, a pinch of boric acid being added as a preservative. During the journey they were as far as possible kept on ice or in cold storage. The results are shown in Table II.

The average vitamin B₁ content was 16.0 μg per 100 ml and none of the samples was particularly poor in the vitamin. In three cases the milk was analysed after the mother had received an injection of vitamin B₁. This did not appear to increase the secretion of the vitamin in the milk.

The injection of 2 mg of pure vitamin B₁ has an almost miraculous effect on acute beriberi in infants. It was anticipated that the breast milk consumed by infants with beriberi would have a very low vitamin B₁ content or be devoid of the vitamin altogether. The results obtained were surprising. They suggest that milk secreted by mothers on a diet very deficient in vitamin B₁ may contain a toxic factor, the product of deranged carbohydrate metabolism, which is absorbed by the infant and can be eliminated only by relatively large doses of vitamin B₁. Japanese workers have been convinced that a toxic factor, akin to methyl-glyoxal, precipitates infantile beriberi. The evidence bearing on this question has recently been discussed by Fehily (1940).

The fact that injection of vitamin B₁ did not increase the secretion of the vitamin in the milk suggests that the additional vitamin B₁ is rapidly absorbed by the maternal tissues and may only pass into the milk after the tissues reach a certain degree of 'saturation'.

TABLE II
The vitamin B₁ content of human milk
(Cases of maternal and infantile beriberi)

Serial number	Age of mother in years	Age of child in days	Condition of child	Treatment of mother with vitamin B ₁	Vitamin B ₁ in μg / 100 ml	Riboflavin in μg / 100 ml	REMARKS
1	25	03	Acute beriberi successfully treated with vitamin B ₁	No treatment	21.8	11.0	The mother had four children previously, all of whom had died from what appeared to be beriberi
2	22	00	No beriberi	For 3 days previously given rice bran and pulses	17.6		Child had taken more thinned milk than breast milk
3	22	01		2 mg of vitamin B ₁ injected on previous day	10.2	30.0	Same case as 2 after treatment of mother with vitamin B ₁
4	10	Child had died 5 days previously at age of 60 days		No treatment	11.8		
5	16			2 mg of vitamin B ₁ by injection on previous day	12.4		Same case as 4 after treatment of mother with vitamin B ₁

TABLE II—*concd.*

Serial number	Age of mother in years	Age of child in days	Condition of child	Treatment of mother with vitamin B ₁	Vitamin B ₁ in μg /100 ml	Riboflavin in μg /100 ml	REMARKS
6	25	14	No beriberi	No treatment	12.4	43.0	
7	26	Twins 7 days old	"	"	10.9	65.0	
8		90	Signs of acute beriberi Treated successfully with vitamin B ₁	"	15.6	44.5	
9		91		10 mg of vitamin B ₁ by injection on previous day	14.6	33.0	Same case as 8 after treatment of mother with vitamin B ₁
10	25	140	Acute beriberi Treated successfully with vitamin B ₁	No treatment	15.0	50.0	
11	30	150	"	"	24.0	68.0	
12	28	120	No beriberi	"	19.2	44.3	
AVERAGE					16.0	48.0	

SUMMARY

1 The vitamin B₁ content of 44 samples of human milk has been determined by the thiochrome method. In this series neither mothers nor infants showed evidence of beriberi. The average vitamin B₁ content was 16.3 μ g per 100 ml when the infant was under 4 months and 19.5 μ g when it was over this age.

2 In cases in which beriberi was present in the mother and in some instances in the child an average value of 16.0 μ g per 100 ml was observed. It is suggested that a toxic factor, the product of deranged carbohydrate metabolism resulting from vitamin B₁ deficiency in the mother, may be the precipitating cause of infantile beriberi.

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PHYSICAL AND CHEMICAL METHODS OF ESTIMATING VITAMIN A IN SHARK AND SAW-FISH LIVER OILS

BY

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SCHEMES have recently been developed in India for producing a cod liver oil substitute from Indian fish liver oils. The liver oil of the shark and saw-fish (*Pristis*) has been found to be considerably richer in vitamin A than cod-liver oil (De, Majumdar and Sundararajan, 1938, Seshan, 1940, Majumdar, 1941). A large amount of oil can often be obtained from a single liver, and the manufacture of medicinal fish liver oils and vitamin A concentrates from these species of fish is a very practical proposition. During the development of these schemes, notably that undertaken by the Department of Fisheries and later the Department of Industries, Government of Madras, the determination of the vitamin A content of a large number of fish liver oil samples was undertaken in the Laboratories. Since it is possible that the production of cod-liver oil substitutes in India may develop into a considerable industry, the methods of estimating vitamin A in these fish oils are of importance. In the present investigation a number of samples was tested by standard spectrophotometric and colorimetric methods and conclusions drawn about the interpretations of the values obtained. All workers are agreed that biological tests for vitamin A are the final and unassailable criterion of vitamin A activity, but they are expensive and time-consuming, and in practice recourse must usually be had to other methods.

EXPERIMENTAL

The majority of samples were supplied through the Director of Fisheries, Madras, or the Superintendent, Kerala Soap Institute, Calicut, where medicinal fish liver oil is now being manufactured. Many of these contained significant amounts of what is usually described as 'stearine', which separates out in cool climate and imparts turbidity to the oil. 'Stearine' is probably a mixture of

various fatty acid glycerides with high melting point. Nearly all the samples were in a fresh condition without objectionable smell.

(i) *Spectrophotometric method*—The vitamin A value of 24 samples of oil was determined by this method. An Adam Hilger's medium quartz spectrograph, in conjunction with a rotary sector photometer, was used. The source of light was a spark obtained between two tungsten-steel electrodes 4 mm apart, by using a high-tension transformer (1 kw) and condenser, the voltage attained on open circuit was about 15,000. Ilford panchromatic plates (S R, H and D 700) were employed. As whole oil was used for the majority of estimations, ether was used as solvent. The turbid samples of oil were saponified and the non-saponifiable matter dissolved in ether. One cm cells were used throughout the investigation and a series of spectra were photographed for each sample for 'density' readings from 0.2 to 1.0. The 'match points' on each pair of spectra were marked with ink dots by visual judgment and the peak of the absorption curve in the region 325 m μ to 328 m μ , obtained by joining the points, was used for the calculation of values. Since 1 cm cells were employed, the density readings directly corresponded with the extinction co-efficient value for the concentration of the solution tested. From this, $E_{1\text{ cm}}^{1\%}$ 328 m μ and international units of vitamin A per gramme, using the conversion factor 1,600, were calculated. A ten per cent reduction in the final value was allowed because ether was used as solvent instead of cyclohexane or absolute alcohol as is the practice in the standard technique. The necessity for this reduction has been pointed out by Lester-Smith, Stern and Young (1938). Gillam and El Ridi (1938) have also found that variations in the extinction co-efficients are obtained with different solvents. The values obtained for the 24 samples of oils are detailed in Table I.

(ii) *Tintometric method*—The same 24 samples were tested by the tintometric method using both untreated and the non-saponifiable matter. As is well known, vitamin A can be recovered quantitatively from the non-saponifiable fraction. A further series of 27 samples was tested by this method alone. The technique of carrying out such tests is well known and it is necessary to mention only a few points.

Saponification—In preparing the non-saponifiable fraction of the oil a slight modification of Edisbury and Morton's (1935) method was used. The ether extraction of vitamin A was repeated at least thrice. The collected ether extract was washed thrice with distilled water and traces of water removed by passing the ether under light suction through an inch thickness of anhydrous sodium sulphate. The sodium sulphate was washed with a little ether and filtered and added to the collected ether extract and evaporated, nitrogen being passed towards the end of the operation to prevent oxidation of vitamin A. The residue in the flask was used for the test with the least delay.

Tintometric test—The concentration of the oil in chloroform (B. P.) was adjusted by repeated trials and dilutions until 0.2 c.c. of the solution (a drop of acetic anhydride was added as a routine), mixed with 2.0 c.c. of a saturated solution of pure antimony trichloride in chloroform (an automatic pipette supplied by B. D. H.

Ltd was used), gave an intensity of blue colour that could be matched between 4 to 6 blue units on the Lovibond scale. A Lovibond tintometer (B D H pattern) and artificial light from the white light cabinet supplied by the manufacturers were used. The readings obtained by the author and by another independent observer agreed within 0.2 unit and were very often correct to 0.1 blue unit. The maximum development of colour was taken as the criterion without relation to any fixed time after addition of the antimony trichloride reagent. Yellow and neutral glasses were frequently used to secure a proper match of colour, but were ignored in the calculation of values. In the case of three samples with a high 'stearine' content and a turbid appearance, red glasses had to be used in addition to blue to get an approximate match in colour. On saponification, however, there was no need for the red glasses. From the concentration of the oil in chloroform in test solution and the blue units required on the Lovibond scale, the proportionate value for blue units for 0.2 c.c. of a 20 per cent strength of the solution was calculated.

Results—Table I gives values for vitamin A of 24 samples of fish liver oils, obtained by the spectrophotometric method. Table II shows the Carr-Price values of the 24 samples tested by the spectrophotometric method, the differences in colour intensity obtained with the non-saponifiable fraction and the whole oil, and the relationship between Carr-Price values and international units. Table III presents data obtained in a further series of 27 samples of fish liver oil by the tintometric method alone. Table III presents vitamin A values in international units per gramme in a further series of 27 samples of fish liver oil calculated by applying the conversion factor 53 (see Discussion) to the values obtained by the tintometric method. The blue values on the whole oil and the non-saponifiable fraction are not given for lack of space.

TABLE I

Vitamin A values obtained by the spectrophotometric method

Number	Concentration of oil tested in mg/100 c.c.	Observed 'density'	$E_{1\text{ cm}}^{1\%}$ 328 m μ .	International units of vitamin A per gramme ($E_{1\text{ cm}}^{1\%}$ 328 m $\mu \times 1,600$) (less 10 per cent)
1	169.0	0.8	4.7	6,768
2	140.0	0.8	5.7	8,208
3	56.0	0.8	14.3	20,592
4	148.0	0.75	5.1	7,344
5	10.5	0.7	66.6	95,904

Samples 1-5—Shark liver oil.

TABLE I—concl'd

Number	Concentration of oil tested in mg /100 c c	Observed 'density'	$E_{1\text{ cm}}^{1\%}$ 328 m μ	International units of vitamin A per gramme ($E_{1\text{ cm}}^{1\%}$ 328 m $\mu \times 1,600$) (less 10 per cent)
6	29.0	0.75	25.9	17,296
7	132.0	0.65	5.0	7,200
8	75.2	0.4	5.3	7,632
9	127.5	0.7	5.5	7,920
10	480.0	0.7	1.5	2,160
11	26.4	0.4	15.2	21,888
12	27.0	0.85	31.5	45,360
13	20.5	0.65	31.7	45,648
14	378.0	0.7	1.9	2,736
15	42.0	0.65	15.5	22,320
16	160.0	0.5	3.1	4,464
17	56.5	0.4	7.1	10,224
18	91.2	0.8	8.8	12,672
19	69.4	0.4	5.8	8,352
20	17.25	0.6	34.8	50,112
21	318.5	0.45	1.4	2,016
22	255.0	1.1	4.3	6,192
23	151.0	0.45	3.0	4,320
24	305.0	0.4	1.3	1,872

Samples 6-19—Shark liver oil
 „ 20-24—Saw fish liver oil

TABLE II
Comparison of values obtained by spectrophotometric and titimetric methods

Number	C P value W O	C P value non sap	I U vitamin A C P value on W O	I U vitamin A C P value on non sap	C-P value W O 1% 328 mμ E ₁ cm	C P value non sap 1% 328 mμ E ₁ cm	C P value non sap C P value W O
1	135	144	50	47	28.7	30.7	1.07
2	162	163	51	50	28.6	28.6	1.01
3	300	350	67	50	21.4	24.5	1.14
4	124	190	50	30	24.3	37.3	1.53
5	1,731	1,673	55	57	20.0	25.1	0.99
6	607	578	61	65	23.5	22.2	0.95
7	85	90	85	73	17.0	19.8	1.17
8	73	110	105	69	14.0	20.8	1.51
9	131	157	90	50	23.8	28.6	1.20
10	17	43	127	50	11.6	28.7	2.50
11	671	651	33	40	44.1	36.2	0.80
12	876	933	52	49	28.0	29.6	1.07
13	918	980	50	46	29.0	31.2	1.08
14	25	52	100	53	13.4	27.4	2.05
15	44.3	385	50	58	28.8	25.0	0.85
16	34	70	131	64	10.8	22.6	2.09
17	147	143	70	71	20.7	20.3	0.98
18	248	243	51	52	28.2	27.6	0.98
19	251	284	33	29	43.3	40.0	1.13
20	1,037	1,063	48	47	29.8	30.6	1.02
21	21	49	101	41	14.3	35.0	2.45
22	32	86	104	72	7.4	20.0	2.69
23	88	106	49	41	29.3	35.3	1.20
24	26	37	72	51	10.0	28.5	1.46
AVERAGE			73	53	23.6	28.5	
W O = Whole oil		Non sap = Non saponifiable matter		I U = International units		C-P value = Carr Price value	

Vitamin A in Shark and Saw-Fish Liver Oils

TABLE III

Values obtained by the titometric method

Number	CP value (non sap) $\times 53$ = I U/g	CP value non sap CP value W O	Number	CP value (non sap) $\times 53$ = I U/g	CP value non sap CP value W O
25	22,260	0.98	39	2,438	2.42
26	1,208	1.13	40	13,833	1.73
27	753	2.15	41	11,872	1.05
28	3,127	3.04	42	1,120	5.43
29	3,074	1.30	43	3,127	1.25
30	1,484	1.81	44	17,278	1.44
31	1,118	1.02	45	5,353	1.46
32	445	2.71	46	9,011	2.34
33	535	1.18	47	2,830	2.16
34	42,612	0.85	48	700	1.25
35	10,070	0.78	49	451	1.88
36	10,865	1.11	50	4,770	1.41
37	212	1.33	51	2,120	2.33
38	2,650	2.89			
			Average of 51 samples	12,067	1.60

Samples 25-42—Shark liver oil
 43-51—Saw fish liver oil
 CP value = Carr Price value

Non sap = Non saponifiable matter
 W O = Whole oil
 I U = International units

DISCUSSION

Though the spectrophotometric method of assay of vitamin A is considered to be accurate, it is necessary to remember certain limitations. The extinction co-efficient for pure vitamin A has been given widely different values by different workers and the conversion factor 1,600, commonly used to express 'E' value in terms of I U, may not be the right figure. If Moll and Reid's (1939) and Grab's (1939) observations are accepted the conversion factor would appear to be significantly different according to whether the saponified or untreated oil is under test. Other possible sources of error may be the presence of cyclized vitamin A which gives an absorption band at 328 m μ but possesses no biological activity, and the unselective absorption at that region given by oxidation products of vitamin A. Vitamin A₂, which has biological activity, has an absorption band quite different from that of vitamin A₁, and this may be a complicating factor. There was no evidence of absorption due to vitamin A₂ in the samples tested.

The tintometric method of estimation has been the subject of much criticism. Poor agreement of colorimetric and biological assay was reported by several workers (Ghosh and Guha, 1935, Morgan, Edisbury and Morton, 1935). Norris and Church (1932) have, however, shown that within a certain range of colour development there is good agreement between colorimetric values and the results of biological assay. In a careful study Coward, Dyer and Morton (1931) found good agreement between Lovibond blue values and biological assay values if the non-saponifiable fraction of the oil was used. In the present investigation both the whole oil and the non-saponifiable fraction were tested and the colour match in the tintometer was made in the region 4 to 6 blue units, where the intensity of colour is assumed to be proportional to the concentration of the oil in solution. The colour development was deeper and brighter when the non-saponifiable fraction was used, the intensity being on the average 1.60 times the colour development with the whole oil. Evers' (1934) figure for this ratio, based on 48 samples of cod-liver oil, was 2.17 and Dyer's (1933) average for 39 samples of cod-liver oil was 1.61. These are in substantial agreement with the ratio obtained in the present investigation. From Tables II and III it is seen that the ratio blue value on non-saponifiable matter/blue value on whole oil is in general near unity for oils having a high blue value (200 C.P. units and above), e.g. oils Nos 5, 6, 11, 12, 13, 15, 18, 20, 25, 34, 35 and 41. There are, however, a few oils in which this relationship does not hold, e.g. oils Nos 3, 17, 19 and 44. Dyer (*loc cit*) observed that blue values determined on the non-saponifiable matter with oils of very high blue value were about the same as that obtained in whole oil. It is possible that the increased intensity of colour obtained with the non-saponifiable fraction is due to removal of 'inhibitors' present in the whole oil in the saponifiable part. Since with oils of high blue value only a small amount of the oil is taken for the test and consequently the amount of 'inhibitors' present is comparatively small, the same blue value is usually obtained both with the whole oil and the non-saponifiable matter (Bacharach and Smith, 1934). Another point is that vitamin A usually occurs in untreated liver oils in the form of esters (Bacharach and Smith, *loc cit*, *Ann Rev Biochem*, 1940), and as free alcohol in the non-saponifiable fraction of the oil. The esters have a higher biological activity

than the alcohol although the former give a less intense colour with the antimony trichloride

It will be thus seen that the expressing of blue values in terms of international units is open to criticism. In addition to the difficulties mentioned above, the factors influencing the accuracy of any method adopted for conversion may vary from species to species and even from sample to sample of oil obtained from the same species of fish. Since, however, an industry for producing cod-liver oil substitutes is now developing in India, the testing of oils is necessary to standardize products. Biological assay is tedious and expensive, and the spectrophotometric method can be carried out only in well-equipped laboratories. In practice, it is probable that the colorimetric test will be largely employed. On the other hand, manufacturers and even the public are now familiar with the expression of the vitamin A value of fish liver oils in terms of international units.

In the circumstances it might be justifiable to use an approximately accurate conversion factor in order to express blue value in terms of international units. Table II shows that when whole oil is used the factor $\frac{I}{B} \frac{U}{U}$ varied enormously; it ranged from 33 to 131, with an exceptional value at 149, and an average of 73. Obviously the use of the factor 73 can scarcely be recommended. There is, however, better agreement when the non-saponifiable fraction of the oil is used. The conversion factor in 24 samples varied from 39 to 73, with one very low value of 29, the average being 53. Recent work by Seshan (*loc cit*) on some Indian fresh-water and marine fish showed that the ratio $\frac{I}{B} \frac{U}{U}$ on non-saponifiable matter was on the average 55, a figure corresponding to that obtained in the present investigation. If for any reason it is desired to express blue values obtained on shark liver oil in international units, a conversion factor of 50 to 55 may not lead to serious error, although dubious from a scientific point of view, provided the blue values are obtained on the non-saponifiable fraction. Lathbury's (1934) figure for this ratio in cod-liver oil was 40 and Evers, Jones and Smith (1936) have reported an average value of 32.9 in the case of halibut liver oils. The relationship between spectrophotometric values and blue values when both the methods of estimation are carried out on the same sample of oil has been expressed by some workers by the ratio —

$$\frac{B}{E} \frac{U}{I} = \frac{328 \text{ m}\mu}{1\% \text{ at } 1 \text{ cm}}$$

Lathbury (*loc cit*) found the value for this ratio in non-saponifiable matter of cod-liver oil to be 27.5. Crews and Cox (1934) found that for fish liver oils of blue value up to 60 the ratio was in the neighbourhood of 30. For oils of high potency—of 600 blue value and above—the figure was nearer 50. Seshan's (*loc cit*) average for Indian fish liver oils was 24.4. In the present investigation, the average value of the ratio was 23.6 for the untreated and 28.5 for the non-saponifiable fraction of shark liver oil respectively.

The average vitamin A activity of the shark and saw-fish liver oil samples was in round figures 12,000 international units per gramme (shark, about 13,600,

saw-fish, about 8,000) This includes both spectrophotometric results and values calculated in international units from Carr-Price values by the conversion factor given above (53) Corresponding figures given by Majumdar (*loc cit*) were 10,000 for shark and 12,000 for saw-fish liver oils The results of the present investigation re-affirm the value of these oils as a potent source of vitamin A

SUMMARY

1 Comparison was made between the values for vitamin A obtained by the spectrophotometric and tintometric methods for 24 samples of shark and saw-fish liver oils

2 The ratio $\frac{\text{international units}}{\text{blue values}}$ was on the average 53 There was less variation in the ratio when non-saponifiable matter was used for the estimations The ratio $\frac{\text{blue value}}{E_{1\%}^{1\text{cm}} \quad 328 \text{ m}\mu}$ was found to be 23.6 and 28.5 for untreated oils and non-saponifiable matter respectively

3 The colour obtained by the antimony trichloride reaction was on the average 1.60 times more intense on the non-saponifiable matter than on whole oil

4 The average vitamin A value was 13,600 and 8,000 international units per gramme for 37 samples of shark and saw-fish liver oils respectively

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AN ADSORPTION METHOD FOR THE ESTIMATION OF NICOTINIC ACID CONTENT OF ANIMAL TISSUES AND BLOOD

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In an earlier paper (Giri and Naganna, 1941) we presented an adsorption method involving the use of medicinal charcoal as adsorbent for the quantitative estimation of nicotinic acid content of foodstuffs. This method has now been applied to the analysis of animal tissues and blood for nicotinic acid.

EXPERIMENTAL

The method adopted for the colorimetric estimation of nicotinic acid was the same as that described by Harris and Raymond (1939)

Reagents required —

- 1 CNBr solution — Prepared by adding 10 per cent aqueous solution of KCN drop by drop to saturated bromine water until it is decolorized
- 2 Amine reagent — One g of p-amino acetophenone is dissolved in 2.8 ml of 10 per cent HCl and diluted to 10 ml. with water
- 3 40 per cent sodium hydroxide
- 4 10 per cent hydrochloric acid
- 5 Acetic acid-acetate buffer M/5 (pH 5.6)
- 6 Alcohol-sodium-hydroxide mixture — Prepared by mixing 20 ml of N NaOH and 80 ml. of absolute alcohol, and making up the volume to 100 ml. It should be prepared fresh when required. The slight turbidity present in the alcohol-NaOH mixture can be removed by centrifuging

7 *Standard solutions of nicotinic acid —*(i) 100 μg per ml (stock solution)(ii) 10 μg per ml (prepared from stock solution once in three days) These solutions were always kept in a refrigerator

8 Medicinal charcoal (Merck)

I ANIMAL TISSUES

The procedure for the extraction, adsorption and elution of nicotinic acid was exactly similar to that described in a previous communication (Giri and Naganna, *loc cit*) Five g of the fresh tissue were used for each determination After adjusting the pH of the final solution to 5.6, the volume is made up to 25 ml with absolute alcohol The final solution is perfectly colourless in all cases In the case of liver, however, the solution is faintly coloured yellow which does not interfere with the colorimetric estimation of nicotinic acid

Estimation of nicotinic acid—Five ml of the solution are taken in graduated glass-stoppered cylinders and 5 ml of acetic acid-acetate buffer M/5 (pH, 5.6) are added to each of the cylinders and kept in water-bath at 80°C for about 10 minutes The cylinders are then removed from the water-bath and 2 ml of CNBr solution are added After cooling the contents by keeping in cold water for about 4 minutes, 0.2 ml of the p-amino-acetophenone reagent is added and kept at room temperature for 15 minutes, the cylinders being covered with black paper to prevent exposure to light Then 0.2 ml of 10 per cent HCl is added and after 15 minutes the colour developed is compared with that of the standard treated similarly The standard solution should be prepared in alcohol-sodium-hydroxide mixture and the pH adjusted to 5.6 after neutralization

Recovery of nicotinic acid added to tissues—Known amounts of nicotinic acid are added to the tissues and the total nicotinic acid is determined The results are presented in Table I The recovery is good in all cases —

TABLE I

Recovery of nicotinic acid added to the tissues.

Tissues	Nicotinic acid in 5 g of tissue, μg	Added nicotinic acid, μg	Total nicotinic acid found, μg	Nicotinic acid recovered, μg	Recovery of added nicotinic acid, per cent
Sheep liver	506	100	597	91	91
Sheep kidney	300	200	500	200	100

Nicotinic acid content of animal tissues — The results from the application of the adsorption method for the estimation of the nicotinic acid content of animal tissues are shown in Table II —

TABLE II

Nicotinic acid content of some organs and tissues

Organs and tissues	NICOTINIC ACID MG /100 G FRESH WEIGHT OF TISSUE			
	Sheep	Cow	Pig	Fowl
Liver	10.1	8.6	16.0	6.2
Kidney	6.0	4.4	7.2	
Spleen	3.8	3.2	5.3	
Heart	4.4	4.9	7.3	2.9
Brain	3.2	2.4	6.4	
Muscle	2.8	4.0	6.2	4.2

The results show that among the tissues liver is the richest source of nicotinic acid. The nicotinic acid content of other tissues is of the same order of magnitude as that of kidney.

II BLOOD

The method has been applied to the analysis of blood for nicotinic acid.

To 10 ml. of newly shed oxalated blood, 30 ml. water are added followed by 5 ml. of 40 per cent sodium-hydroxide solution. The mixture is kept in a boiling water-bath for about 30 minutes in order to convert the nicotinamide into nicotinic acid. It is then neutralized with concentrated HCl and brought to pH 5.0 (yellow to bromo-cresol purple) by the addition of dilute HCl. It is then centrifuged to remove the precipitate formed after neutralization, and the supernatant liquid is decanted.

The procedure for the repeated adsorption of nicotinic acid from the supernatant liquid and the elution with alcohol-sodium-hydroxide mixture is exactly similar to that described for animal tissues. After adjusting the pH of the final solution to 5.6, the volume is made up to 20 ml. with absolute alcohol. The solution possesses faint yellow colour. Occasionally after the first centrifugation during the elution, the eluate still contains traces of carbon particles, which impart light dark shade to the solution. This can be removed completely by again centrifuging in 10 ml. centrifuge tubes after decantation, when a perfectly clear solution is obtained.

For colorimetric estimation of nicotinic acid, 10 ml. of the experimental solution are taken in graduated glass-stoppered cylinder and 5 ml. of acetic acid-acetate

buffer (pH 5.6) are added. The order of the addition of reagents and the procedure for the development of the colour are exactly the same as those described for animal tissues.

TABLE III.

Recovery of added nicotinic acid to blood

Blood	Nicotinic acid of blood sample in 10 ml, μg	Added nicotinic acid, μg	Total nicotinic acid as determined, μg	Nicotinic acid recovered, μg	Recovery of added nicotinic acid, per cent
Sheep blood	71.5	200	244	173	87
Human blood	18	100	123	105	105

The figures show that the recovery of added nicotinic acid to sheep and human blood is quite satisfactory.

Nicotinic acid content of human blood in normal, pellagra and leprosy—The blood of normal persons, and of pellagrins and lepers were then examined for their nicotinic acid content and the results are given in Table IV—

TABLE IV

Nicotinic acid content of human blood in normal, pellagra and leprosy

Blood in		Nicotinic acid per 100 ml of blood
		mg
1	Normal	0.383
2	Normal	0.360
3	Normal	0.330
4	Normal	0.380
5	Pellagra	0.190
6	Leprosy type N ₂	0.200
7	Leprosy type C ₁ N ₂	0.204

It is interesting to note that the blood of lepers and pellagrins contain less of nicotinic acid than that of normal persons. It would be, however, premature to base any wide conclusions upon the limited findings reported here.

DISCUSSION

Values for the nicotinic acid content of animal tissues have been reported by Swaminathan (1938) for sheep's liver, by Bandier (1939) for pig ox and cod tissues, and by Kodicek (1940) for sheep and ox tissues. The data that have been gathered indicate that the amount of nicotinic acid in liver is 11 mg to 20 mg, kidney 6.5 mg to 7.5 mg, spleen 4.0 mg to 4.4 mg, brain 3.0 mg, and heart muscle 5.3 mg to 5.9 mg per 100 g of the tissue. The results obtained in the present investigation by the application of our adsorption method are of the same order as those obtained by other workers. The values obtained for the nicotinic acid content of normal blood are of the same order as those given by Swaminathan (*loc cit*) and Kochhar (1940).

While our previous paper (Giri and Naganna, *loc cit*), describing the adsorption method, was in press, the report by Melnick and Field (1940) appeared, in which a method for the determination of nicotinic acid in biological materials has been described. The authors found that charcoal adsorbs nicotinic acid quantitatively from pure aqueous solutions, which is in complete accord with the observations made in our previous communication (Giri and Naganna, *loc cit*) on the subject. They also found that charcoal adsorbs nicotinic acid to an appreciable extent from biological solutions. They have, however, made use of preferential charcoal adsorption for the decolorization of solutions under conditions in which nicotinic acid is not adsorbed by charcoal, i.e. in presence of alcohol, a fact which has already been established in our previous communication.

SUMMARY

An adsorption method for the determination of the nicotinic acid content of animal tissues and blood is described. Results are given of a series of analysis of sheep, cow, pig and fowl tissues, and the blood of normal persons, pellagrins and lepers.

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ASSESSMENT OF VITAMIN A DEFICIENCY AMONGST
BENGALEES AND DETERMINATION OF THE
MINIMAL AND OPTIMAL REQUIREMENTS
OF VITAMIN A BY A SIMPLIFIED
METHOD FOR MEASURING
VISUAL ADAPTATION
IN THE DARK

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THE determination of vitamin A deficiency amongst humans by the measurement of their visual adaptation in the dark is based on theoretical considerations which were discussed by various authors (Hecht, 1919, 1921, 1937, Hecht and Schaefer, 1938, Hecht and Mandelbaum, 1939, Lythgoe, 1937, 1938, 1940, Dartnall *et al*, 1937, Wald, 1934, Jeans, Blanchard and Zentmire, 1937). The concentration of visual purple, of which the regeneration in the dark after bleaching forms the basis of dark adaptation, has been found to increase in the retina of cats from 57 to 95 per cent of its final value between the 10th and 30th minute after dark adaptation begins (Granit *et al*, 1939). If this be true also for human beings, the sensitivity of the retina may, according to this rule, be expected to be nearly doubled, although, in fact, it is increased 100 times or 1,000 times or even much more. Granit *et al* (*loc cit*) suggest that this extraordinarily large increase in the sensitivity of the retina is probably due to a nervous interaction. The possibility that the visual purple at higher concentrations may cause a discharge of volleys of impulses of much greater frequency and for a longer period has not been ruled out. Thus, the enormous increase in sensitivity of the retina during dark adaptation may still be explicable on the basis of a change in concentration of visual purple.

The instruments which are generally used for measuring dark adaptation are Birch-Hirschfeld photometer, biophotometer by Jeans *et al* (*loc cit*), Hecht and Schaefer (*loc cit*) adaptometer, Pett's (1939) photometer and Rowett adaptometer by Thomson *et al* (1939). These instruments differ widely not only in the principle of their construction but also in their use for measuring dark adaptation. In the course of the determination of the curve of dark adaptation Hecht first noticed that the curve is dual in nature with a kink at the intersection of two parts. The first part of the curve is over in the course of 7 minutes after the bleaching light is switched off, and during this period the flashes of light are recognized by the subject as coloured, while those in the second part are colourless. He, therefore, concluded that the first part of the curve is caused mainly by the adaptation of the cones and the second part mainly by that of the rods. It was also noticed that the rate of restoration of visual acuity due to cone mechanism (i.e. scotopic mechanism) is different from that due to rod mechanism (i.e. photopic mechanism) although vitamin A is found to be just as essential for the restoration of cone vision as for rod vision (Lythgoe, 1940, Hecht and Schaefer, *loc cit*).

There are two views about the criteria by which the impaired power of dark adaptation, as found in cases of vitamin A deficiency, may be judged. These are —

(a) It is the rate of dark adaptation that is affected and that every one ultimately reaches the same light threshold when completely dark adapted.

(b) The light threshold of the completely dark-adapted eye is appreciably changed during vitamin A deficiency. Booher *et al* (1939) pointed out that, although the curves of dark adaptation of deficient persons are inferior to those of normal persons at every point during a period of 30 minutes or longer, the differences are far more obvious at the end than at the beginning. Wald, Jeghers and Arminio (1938) showed that in cases of acute vitamin A deficiency the light threshold at complete dark adaptation is 50 times greater than that of a person taking a normal diet with a supplement of vitamin A concentrate. It is thus obvious that readings taken after a short period of dark adaptation may not show the extent of deficiency of vitamin A or real impairment of dark adaptation. It has also been shown by Thomson *et al* (*loc cit*) that a preliminary dark adaptation of 10 minutes followed by bleaching, is inadequate to eliminate the effect of previous light, and the methods in which this point is not taken into consideration cannot be expected to give constant results. It is evident from these observations that Birch-Hirschfeld's or Jeans' or Pett's method is not likely to give consistent results. Further, as has been shown by Hecht and Schaefer (*loc cit*), indirect fixation, 7° nasally, to a small test field lighted momentarily, is expected to give better results than direct fixation of the test field, as is done in these three methods.

EXPERIMENTAL

The present investigation was commenced with Jeans' biophotometer. Simultaneously with the measurement of dark adaptation of the subjects, their daily diet was recorded and its vitamin A or carotene content calculated from the

available analytical charts. The technique followed was exactly in accordance with the directions of the authors. After a preliminary rest in a completely dark room for 10 minutes, the eyes of the subject were exposed to a standardized bright light for exactly 3 minutes. The restoration of his vision in the dark was then studied by his power of perception of a dim light of known but variable intensity flashed before his eyes, first 20 seconds after the extinction of the light, and then after every 2 or 3 minutes and finally at the end of 10 minutes. The intensities of light just perceived during these trials are mentioned as D R (dial reading) values*.

The results of examination are shown in Table I. A few typical results are plotted out as dark-adaptation curves (*vide* Graph 1).

TABLE I

Showing the data of dark-adaptation studies on 39 subjects by Jeans' biophotometer

Number of subjects	Age in years.	BIOPHOTOMETER DIAL READINGS (D R)			Remarks about the vitamin A content* of the diet consumed by the subjects
		D R after the preliminary dark adaptation for 10 minutes	D R. at 20 seconds after the bleaching light was switched off	D R at 10 minutes after the bleaching light was switched off, i.e. after dark adaptation for 10 minutes	
1	29	70	30	79	Good.
2	25	80	27	73	Bad
3	19	69	30	75	Good
4	18	72	12	65	Bad
5	22	70	20	75	Good
6	19	80	20	83	"
7	19	75	26	74	"
8	21	79	28	75	"
9	21	75	35	75	"
10	19	70	20	68	Very good
11	18	60	25	75	Good.
12	16	53	22	62	Very good
13	37	65	30	71	"

* Very good diet — vitamin A intake roughly 4,000 to 6,000 I. U. daily
 Good diet — " " " 3,000 to 4,000 " "
 Not good diet — " " " 2,000 to 3,000 " "
 Bad diet — " " " 1,000 " "
 Very bad diet — " " " 200 to 500 " "
 Vegetarian diet — " " " 500

* In the instrument there is a dial by the movement of which the intensity of the flashing light is increased or decreased. The movements of the dial are made against a scale of which the higher values correspond to lower intensities of light and vice versa. These values are mentioned as D R values. In the various tables annexed herewith the D R values have also been expressed in both millifoot candle and log micro micro lambert.

TABLE I—concl'd

Number of subjects	Age in years	BIOPHOTOMETRIC DIAL READINGS (D R)			Remarks about the vitamin A content* of the diet consumed by the subjects
		D R after the preliminary dark adaptation for 10 minutes	D R at 20 seconds after the bleaching light was switched off	D R at 10 minutes after the bleaching light was switched off, i.e. after dark adaptation for 10 minutes	
14	16	64	9	53	Good (vegetarian)
15	17	60	25	59	Good
16	17	80	28	76	"
17	20	58	28	65	"
18	22	80	20	76	Good (hostel diet)
19	48	55	8	48	Good
20	25	80	12	74	Bad
21	32	65	25	68	"
22	40	60	10	52	Good
23	21	75	20	80	"
24	50	70	22	70	Not good
25	39	60	5	52	Bad
26	32	50	20	72	"
27	37	62	25	65	"
28	26	70	12	62	"
29	29	65	25	63	"
30	40	50	5	44	"
31	30	55	20	55	"
32	40	45	4	36	Very bad
33	48	54	20	60	Good
34	35	70	30	76	"
35	20	70	25	72	"
36	24	70	25	67	"
37	24	60	12	55	Bad
38	23	47	16	62	"
39	29	80	35	82	Good

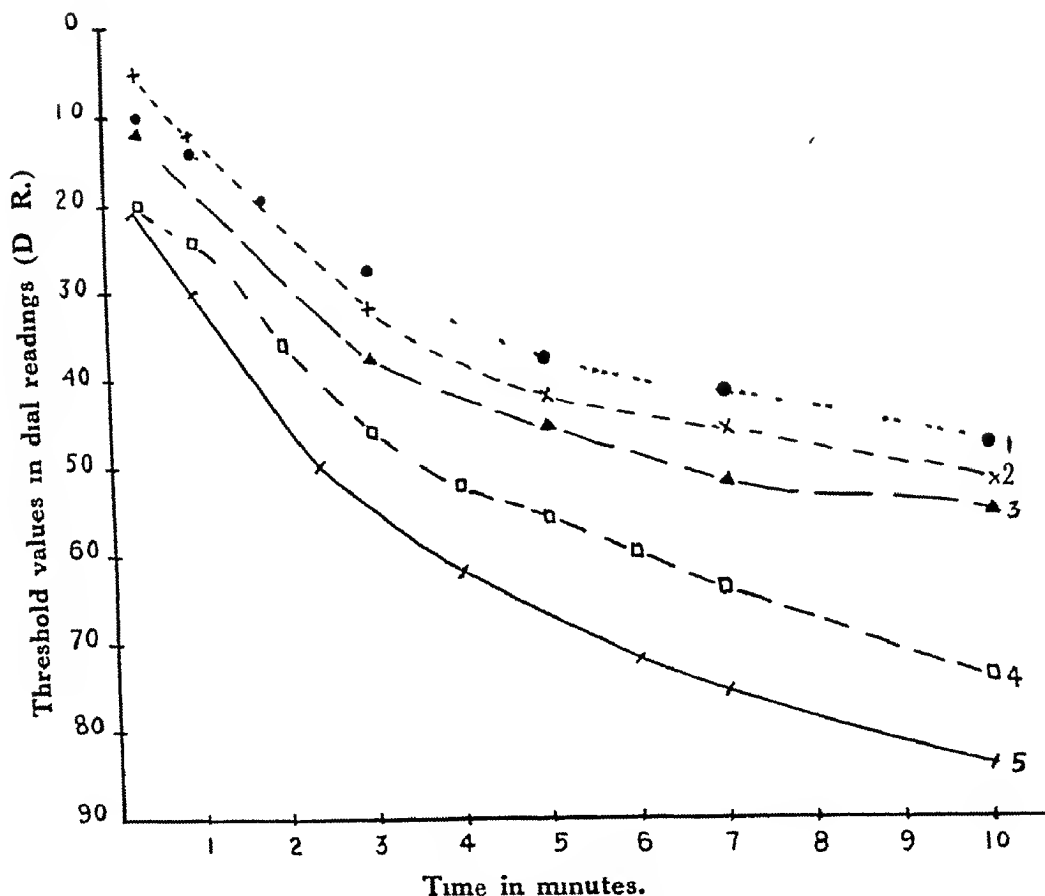
* Very good diet — vitamin A intake roughly 4,000 to 6,000 I U daily
 Good diet — " " " 3,000 to 4,000 " "
 Not good diet — " " " 2,000 to 3,000 " "
 Bad diet — " " " 1,000 " "
 Very bad diet — " " " 200 to 500 " "
 Vegetarian diet — " " " 500 " "

DISCUSSION OF RESULTS

(a) According to Jeans *et al* (*loc cit*) the light-adaptation values, at 20 seconds and at 10 minutes after the extinction of the bleaching light, are particularly important as these indicate the vitamin A content of the body more than the other observed values during the period of the experiment. The normal adaptation values at these periods should, according to these authors, be 1.0 and 0.1 millifoot candles (mfc) respectively (equivalent to 14 and 43 in the scale of the dial attached to the instrument).

GRAPH 1

Showing some typical dark-adaptation curves obtained by Jeans' method



Curves 1, 2 and 3—subjects deficient in vitamin A.
Curves 4 and 5—normal subjects.

Table I shows that out of the 39 cases examined (with no definite eye lesion), 10 persons gave D R values at 20 seconds less than the normal figure of 14, while only one subject gave a figure (= 36) less than the normal figure of 43 at 10 minutes. The incidence of vitamin A deficiency is, therefore, either 25.6 per cent or 2.6 per cent according as the D R value at 20 seconds or at 10 minutes be taken to indicate the vitamin A content of the body. As the figures differ widely, it follows that the observed values in either of these periods may not represent the true vitamin A status of a person.

(b) In 12 cases out of the 39, the dial readings obtained did not correspond with the vitamin A intake in their diet (cf Table I).

(c) The preliminary dark adaptation of 10 minutes was not sufficient to bring the various subjects to a uniform condition of dark adaptation of their eyes. This is obvious from Graph 4 in which it will be seen that the light-threshold values of the same person under the same conditions of the dietary intake of vitamin A, at 10 minutes after dark adaptation are widely different under different conditions of the pre-adapting light. This question has been more fully discussed in connection with the defects of Hecht's methods.

(d) Subsequent observations for 10 minutes in the dark may fail to reveal the actual difference in the dark-adaptation curve of a deficient person from normal, as this difference may become more evident after 10 minutes (*cf.* observations of Booher *et al.*, 1939, and Wald *et al.*, 1938).

It is obvious from these observations that Jeans' instrument and technique cannot be expected to give consistent results about the vitamin A status of persons. Palmer *et al.* (1938), Girdgman and Wilkinson (1938), and Isacs *et al.* (1938) have also criticized the methods of Jeans *et al.* and Birch-Hirschfeld.

MODIFICATION OF THE INSTRUMENT

As Hecht's (*loc. cit.*) instrument appears to be most satisfactory from theoretical considerations, the biophotometer was modified in accordance with the principles of this instrument. The quincunx in the shutter of Jeans' instrument was replaced by a camera diaphragm of approximately 4 cm diameter. A thick black cardboard was pasted on the back of the diaphragm, i.e. in the direction of the lamp. There were two holes of different diameters in this cardboard separated by a distance of 2.95 cm. The diameter of the larger hole was 1.26 cm. It was covered by a piece of white paper of nearly the same quality as that found at the back of the dot in the middle of the quincunx so that the light intensity of this field was made as nearly as possible equal to that shown by the middle dot of the quincunx. This light area constituted the test field in the modified instrument. The other hole (uncovered) had a diameter of 0.5 cm and constituted the fixation point. The diameter of the larger hole was so chosen as to correspond to Hecht's test field, 3° in diameter. Its distance and direction from the other hole were such that when the smaller hole was fixated upon by the right eye, the former hole had its image focused 7° nasally on the retina where the populations of the rods and cones are nearly equal. The lever of the camera-diaphragm can be worked from outside so quickly that flashes of light can be given in about 1/5th of a second. As coloured glasses of the specification recommended by Hecht could not be procured, white test light was used.

FURTHER EXAMINATION OF ANOTHER BATCH OF SUBJECTS BY THE MODIFIED INSTRUMENT IN ACCORDANCE WITH HECHT'S METHOD

Fifty-four subjects apparently of normal health and vision were examined by this modified instrument in accordance with the technique recommended by Hecht. The dark-adaptation values of the subjects, after their eyes were exposed to a bleaching light for exactly 3 minutes, were recorded in a completely dark room at the

following intervals, viz $\frac{1}{2}$ minute, 1 minute, 3 minutes, 5 minutes, 6 minutes, 7 minutes, 8 minutes, 9 minutes, 10 minutes, 15 minutes, 20 minutes, 25 minutes and 30 minutes. The data about food intake by various persons examined were collected in the following way: each subject was given a diet card to be filled up as per instructions given therein. In the case of boys these cards were filled up by their guardians who were requested to give a fairly approximate proportion of the various foods consumed by their wards under examination. The average vitamin A intake *per diem* was calculated from their daily food consumption for a period of a week or so.

Results of examinations—These are given in Table II and Graphs 2 and 3

TABLE II

The frequency distribution of 54 apparently normal subjects with regard to their final light-threshold values measured according to the principles of Hecht

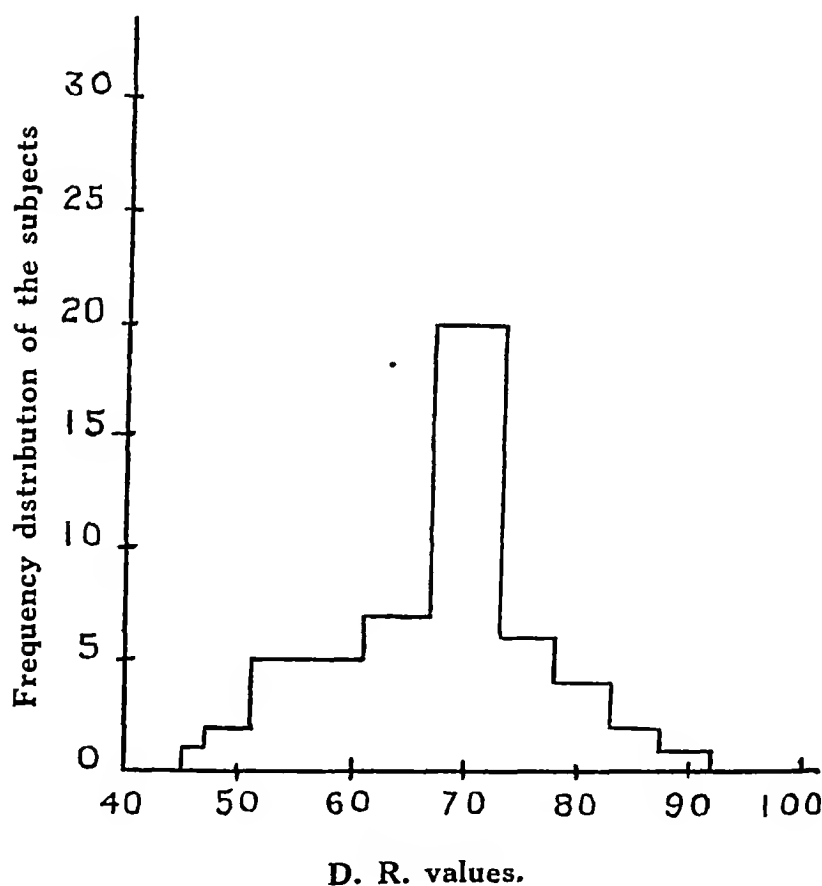
THRESHOLD VALUES THIRTY MINUTES AFTER THE STANDARD LIGHT ADAPTATION			Frequency distribution according to final threshold values.
In dial reading (D R)	In millifoot candles	Log micro micro lambert.*	
90	0.0023	3.3617	1
85	0.0035	3.5441	2
80	0.0051	3.7076	4
75	0.0077	3.8805	6
70	0.0114	4.0569	21
65	0.0170	4.2304	7
60	0.0252	4.4014	5
55	0.0375	4.5740	5
50	0.0557	4.7459	2
45	0.0767	4.9281	1

* 1 micro-lambert = 1 millifoot candle

Table II shows the distribution of the subjects with regard to their final light perception values during dark adaptation, obtained at exactly 30 minutes after the bleaching light was switched off and expressed in a gradually descending scale of

GRAPH 2

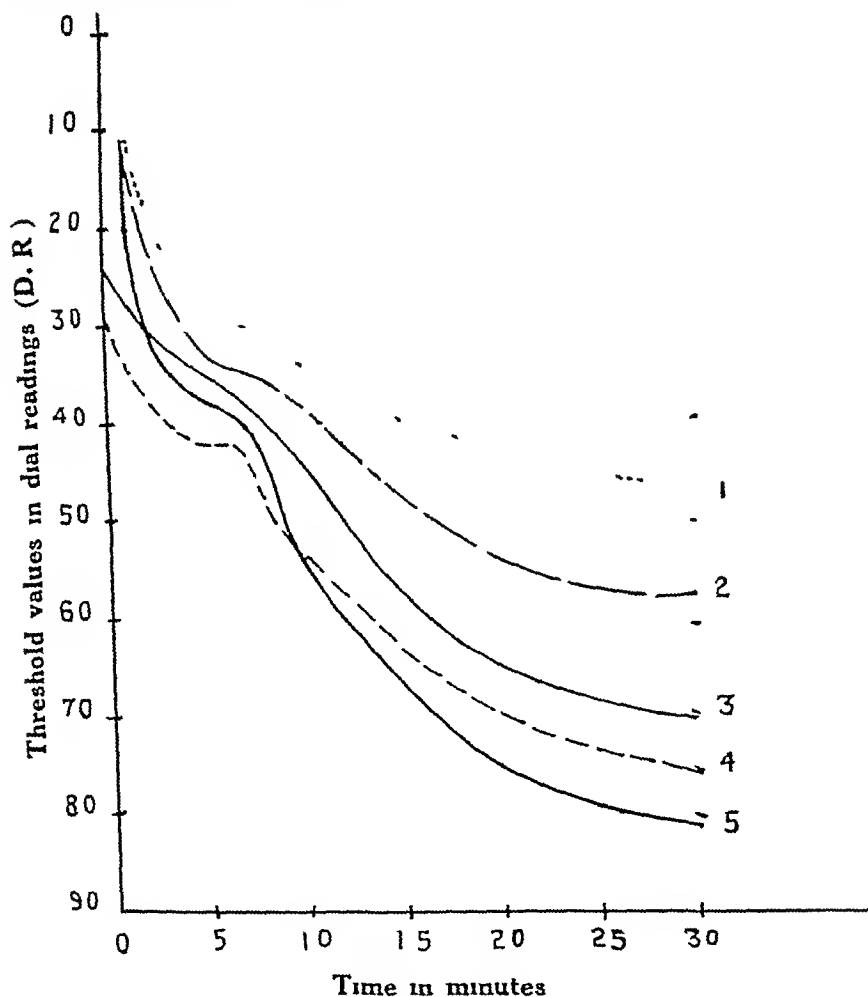
Showing the frequency distribution of 54 subjects with regard to their final light-threshold values determined according to Hecht's method (cf Table II)



dial readings and Graph 2 shows graphically the same results. Graph 3 is a record of some dark-adaptation curves which are considered to be typical of normal and deficient persons.

GRAPH 3

Showing some typical dark-adaptation curves obtained by Hecht's method



Curves 1, 2 and 3—subjects deficient in vitamin A
Curves 4 and 5—normal subjects

DISCUSSION OF RESULTS

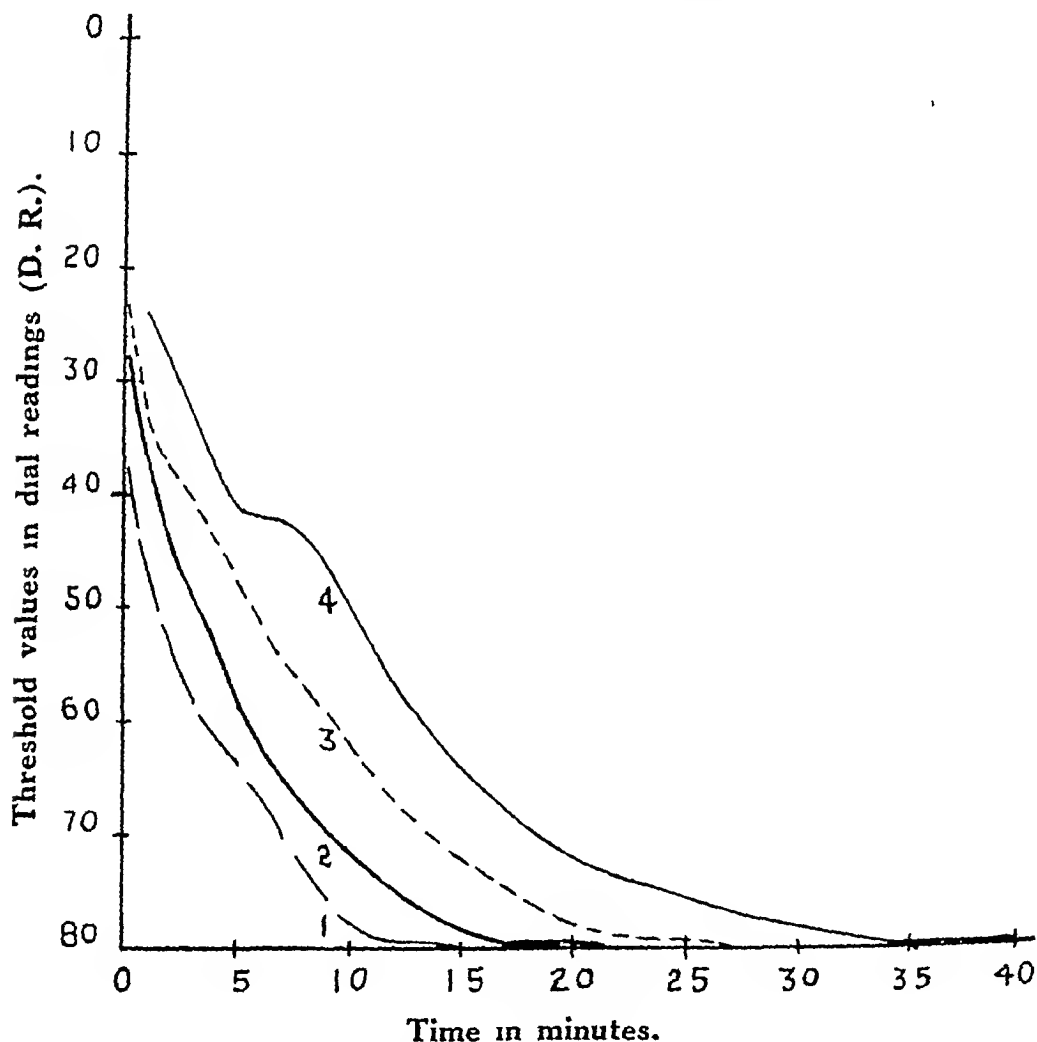
(i) The kink at the point of intersection between the cone and the rod-adaptation parts of these dark-adaptation curves was evident in 28 cases only (i.e. in roughly 50 per cent cases) and appeared about 7 minutes after the bleaching light was switched off

The absence of kink in most cases was probably due to two reasons —

(a) If a coloured test patch had been used, the disappearance of colour which marks the termination of cone adaptation, could have been easily recognized

GRAPH 4

Showing the effect of pre-adaptation light on the visual threshold values of light during dark adaptation



- (1) A subject examined immediately on coming from outside on a rainy day
- (2) The same subject examined as before on coming from shades on a bright sunny day
- (3) The same subject after coming directly from bright sunlight
- (4) The same subject after his eyes are exposed to the bleaching light for 3 minutes

(b) The kink probably lay in the interim period of examination of the eyes with the test light and thus evaded the notice of the observer

(ii) In 5 persons who were suffering from diseases with involvement of the liver, such as hepatitis, jaundice, etc the kink appeared in the following intervals since the commencement of dark adaptation —

- (a) After 8 minutes in 2 cases
- (b) „ 9 „ in 1 case
- (c) „ 10 „ in 2 cases

As the normal interval at which the kink appears is 7 minutes, it may be concluded that liver troubles delay its appearance. Hecht and Mandelbaum (*loc cit*) also observed that while the cone-rod transition time remains unaltered during vitamin A deficiency it is delayed in cirrhosis of liver. Thus, the lengthening of the period of cone-rod transition in lesions of liver may prove to be a valuable aid to diagnosis of such cases.

(iii) In comparing the vitamin A (and carotene) intake of these persons with their light perception values at 30 minutes after the extinction of the bleaching light, it was noticed that there was correspondence between them in 33 cases (out of 54). Thus, a person taking daily about 1,000 I U of vitamin A showed a D R of 56 [= 0.035 millifoot candle (m f c)]. On his taking one cup of milk and 2 eggs daily for 7 days in excess of his diet, his D R rose to 65 (= 0.017 m f c). Again a person consuming roughly about 3,000 I U of the vitamin daily, showed a D R of 70 (= 0.011 m f c) and another taking daily over 4,000 I U gave a D R of 85 (= 0.0035 m f c).

There was no correlation in 21 cases between their D R values and the vitamin A intake. Some of these subjects were either suffering from dyspepsia or had a previous attack of typhoid or kala-azar.

(iv) It will be observed from Table II that a majority of the subjects, viz 63 per cent, gave a D R value of 70 and above. If this D R value, viz 70, be provisionally taken as the minimum final light-threshold value of a normal person, then there is a deficiency of 37 per cent amongst the subjects. As out of these 54 persons 30 were college students (age 17 to 24 years) and 24 were employees (age 18 to 50 years) of moderate income, and as 9 students and 11 employees had D R values below 70, the percentage of deficiency amongst the students and employees comes up to 30 and 46 respectively.

Hecht, on using violet light in the test field, took 80 as the minimum D R value of a normal adult. The acceptance of a lower D R value, viz 70, as the minimal value with this instrument, is justified as white light which has a much lower photochemical activity in the dark than blue or violet light, has been used in the test field of the modified instrument.

DEFECTS OF HECHT'S METHOD AS EMPLOYED

(i) No cognizance was taken of the light-adapted condition of the eye before it is exposed to the bleaching light. Matthey (1933) demonstrated that a full preliminary dark adaptation is required to ensure a standard curve independent of previous light. Thomson *et al* (*loc cit*) have shown (a) that without any preliminary dark adaptation before experimental bleaching the dark-adaptation curve is

not only not constant for the same individual but varies very widely from that obtained after full preliminary dark adaptation and (b) that a preliminary dark adaptation of 10 minutes followed by bleaching is inadequate to eliminate the effects of previous light (*vide* also discussion of the results obtained by Jeans' instrument)

(ii) It is doubtful, if 4 minutes' exposure of the eyes to a strong but bearable light can completely bleach the visual purple of the retina and thus bring the retina to a uniform condition before the dark-adaptation study begins. It has been shown by Lythgoe (1940) that in animals, such as rats, frogs and cats, whether the visual purple is protected or not from light by the migrating pigment epithelium, considerable quantities of visual purple still remain unbleached after their eyes are exposed to an illumination of 1,000 m c (metre candle) for a considerable length of time

(iii) As the time required for the examination of a single person is nearly three-quarters of an hour, it is unsuitable for the assessment of a large number of subjects

Necessity for an accurate but quick method for assessing vitamin A deficiency by visual-adaptation tests—In view of the defects in the techniques employed by Jeans *et al* and Hecht *et al* necessity was felt for the evolution of a quick yet accurate method for determining visual adaptation. Thomson's method of bringing the subject's eyes to a uniform condition of dark adaptation before they are exposed to the bleaching light appears to be theoretically sound, and constant light threshold values in the dark were also obtained thereby from the same person. Curiously enough, Thomson *et al*, by application of their method, came to the conclusion that 'neither the rate of dark adaptation nor the light threshold of the fully dark-adapted eye has necessarily a close correlation with the intake of vitamin A in the diet'—a conclusion which is definitely contrary to the findings of numerous previous workers on the subject and of the authors as well

This strange conclusion is probably due to certain defects in their instrument. These are —

(a) The areas of the test fields in Thomson's and Hecht's instruments are 38.2 sq cm and 1.2 sq cm respectively. The effect on the retina of the illumination of the former test field when seen through aperture No. 2 of that instrument (*vide supra*), which is the standard threshold in that instrument, is expected to be $38.2/1.2$, i.e. 32 times greater than that of the latter, for Aubert noticed 'that the threshold value of a feeble light stimulus varied inversely as the area stimulated' (Greenwood, 1910). Accordingly, the standard threshold in Thomson's instrument may be considered to have the effect of an illumination equivalent to $0.0021 \times 32 = 0.0672$ m f c and not 0.0021 m f c, as mentioned by Thomson (*loc cit*). This intensity of light, viz. 0.0672 m f c, corresponds to a D R value between 46 and 50 (Table II) and was perceived during complete dark adaptation by a large majority of vitamin A deficient persons examined by the authors. It is thus evident that Thomson's technique failed to identify the deficient cases and it is not, therefore, surprising that he was led to the strange conclusion about the absence of correlation between the vitamin A intake and the light perception in the dark.

(b) It may also be pointed out that Thomson *et al* did not examine a sufficient number of deficient persons so as to justify their conclusion which conflicts with the findings of a number of previous workers, such as Frandsen (1937), Toverud (1937), Booher *et al* (1939, 1940), Gridgman and Wilkinson (*loc cit*), McKenzie (1938, 1939), Hecht and Schaefer (*loc cit*), Hecht and Mandelbaum (*loc cit*), Wald *et al* (1938), Pett (*loc cit*) and Tansley (1939)

It, therefore, appeared to the authors that if Thomson's method of bringing the eyes of subjects to a uniform condition of pre-adaptation, as previously referred to, be adopted and Hecht's instrument be employed for determining the visual adaptation, accurate results may be expected. Hecht and Mandelbaum (*loc cit*) have emphasized the value of plotting the whole curve instead of determining an isolated point on it, such as the final cone threshold, cone-rod transition time, final rod threshold and the time required for reaching the latter. The importance of plotting the whole curve is obvious from the fact that there are two variables in the dark-adaptation curve, viz the rate of restoration of the visual perception, i.e. $\frac{dI}{dt}$, at any time and the rod-threshold value at that time (i.e. I), and that these variables may have different values in different normal persons and may be differently affected in different deficient persons. Thomson *et al* (*loc cit*) and others have pointed out that the determination of the first variable, i.e. $\frac{dI}{dt}$, by a simple assessment of the time for reaching a given threshold, does not give constant results. Since both I and $\frac{dI}{dt}$ vary throughout the course of dark adaptation, a rigorous method should include the measurement of both these variables throughout the whole course of the dark adaptation. The expression $\int_{I_0}^I \int_0^t f(I_t) \frac{dI}{dt} dt$ * representing the area of the surface between the curve of dark adaptation and the two axes, seems to indicate a true criterion of the vitamin A status, provided the dark-adaptation curve is drawn after the eyes are first brought to a state of equilibrium, i.e. $\frac{dI}{dt} = 0$, and are then exposed to the bleaching light.

The great disadvantage of this rigorous procedure is that it would take 20 to 25 minutes for pre-adaptation, 4 minutes' exposure to bleaching light and 30 to 45 minutes for the subsequent dark adaptation, i.e. from 55 to 75 minutes for the examination of each subject. This is an unusually long procedure which is absolutely unsuitable for large scale assessment of vitamin A deficiency. It is, therefore, desirable that a much quicker but fairly accurate method be evolved

* I_0 indicates the threshold value at the beginning of the dark adaptation, i.e. immediately after the bleaching light is switched off, I_t the threshold value on complete dark adaptation, i.e. when $\frac{dI}{dt}$ is again zero, and t = the minimum time to reach complete dark adaptation.

and that the tedium of sitting alone in the dark room be dispelled so that the illiterate people may be easily induced to subject themselves to this examination

A simple, quick but fairly accurate method—In evolving such a method the question has to be considered which of the two variables mentioned above is more important and shows more pronounced changes under different conditions of vitamin A saturation of the body. Booher *et al* (1939) have already pointed out that the determination of the second variable, i.e. the final rod-threshold value, is more important and a reference has already been made as to the remarks of Thomson *et al* (*loc cit*) about the determination of the first variable. It is therefore expected that the measurement of the threshold value at complete dark adaptation will give fairly satisfactory and comparative results.

In determining this value alone the preliminary dark adaptation and the exposure thereafter of the eyes to the bleaching light are unnecessary for, as is shown below, they are related to the determination of $\frac{dI}{dt}$ and not of I .

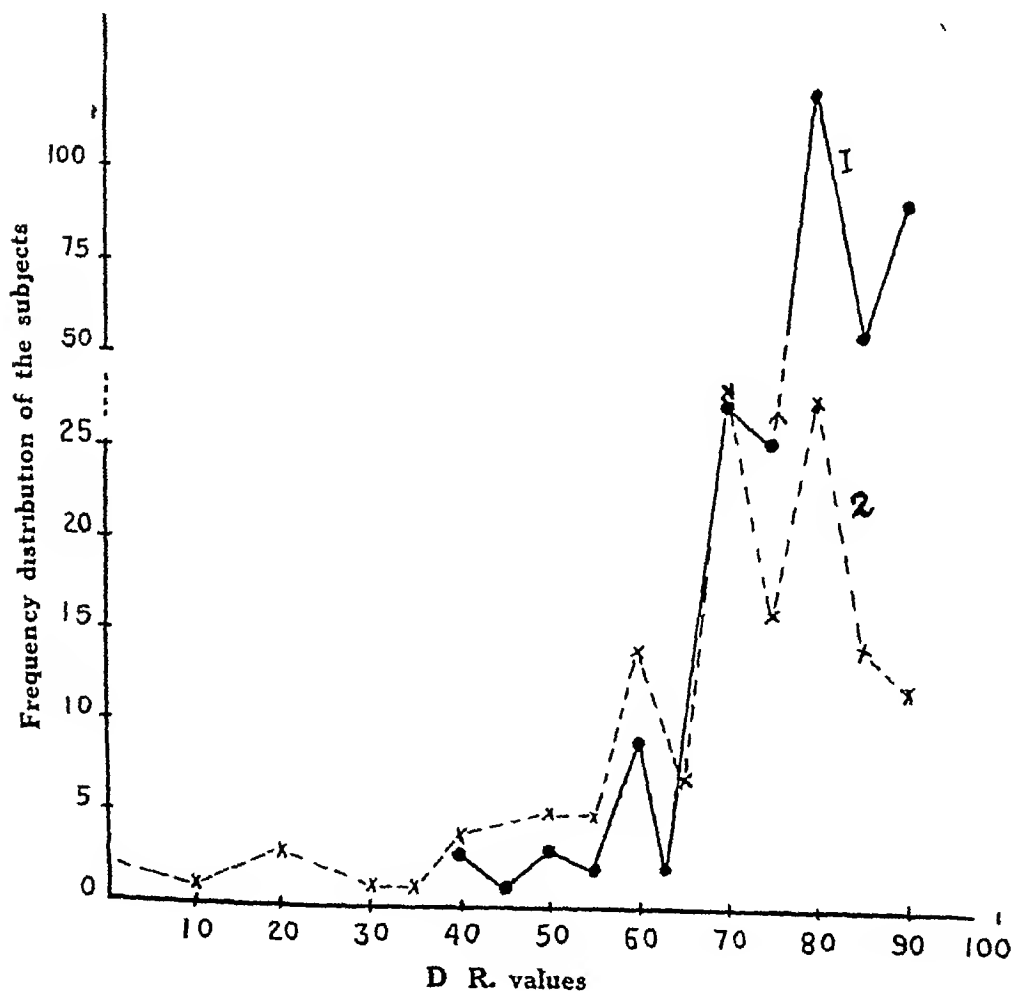
The preliminary dark adaptation in accordance with Thomson's method is expected to bring the retina of the subjects to a uniform condition of power of vision in the dark, i.e. to bring the concentrations of visual purple in the retina of various subjects to the same level, for it has been assumed that the power of visual perception of retina in the dark is a function of concentration of visual purple. If the retina be now exposed to a light of uniform intensity for the same period in the case of all subjects, the photochemical dissociation or decomposition of visual purple is justifiably expected to be the same in all cases. In the following period of darkness, the velocity of regeneration of visual purple ($\frac{dI}{dt}$) is expected to be proportional to the supply of vitamin A in the retina, the concentrations of other substances required in this regeneration remaining necessarily the same in all subjects as they are amongst the products of photochemical decomposition of visual purple (Wald *et al*, 1937-38, Lythgoe, 1940). It is thus obvious that for ascertaining the vitamin A reserve in the body of a person and comparing it with that of another by the determination of the rate of restoration of visual perception (i.e. of $\frac{dI}{dt}$), a uniform initial dark adaptation, followed by the bleaching of the retina to the same extent, is essential. But in the determination of the final threshold value which would naturally depend on the total quantity of visual purple in rods, two factors which would profoundly influence the latter are the period of stay in darkness and the total available amount of vitamin A in the retina. Accordingly, the preliminary dark adaptation and exposure to bleaching light are not necessary and have accordingly been dispensed with.

Technique of the new method—A batch of 10 to 12 persons are taken directly to the dark room and are examined immediately one after the other with the modified instrument. The purpose of this examination is just to make the subjects familiar with the instrument. Without exposing their eyes to the bleaching light, the examination of their eyes was conducted from time to time till each subject

reaches a steady state with regard to his power of perception. This final threshold value is then noted. The time required by the different subjects to reach a steady threshold is found to vary from 15 to 30 minutes.

GRAPH 5

Showing the frequency distribution with regard to final threshold values of 341 students (curve 1) and 161 employees (curve 2), all apparently of normal health and vision (cf Table III)



Results of examination by the new method—The results are shown in Table III which gives the frequency distribution with regard to final light-threshold values amongst the different groups of subjects examined, viz 341 students of three

different schools and 161 employees of moderate income. One of the three schools from which the students were selected for examination is a free school for poor students who cannot afford to pay their tuition fees.

TABLE III

*Showing the frequency distribution according to the final rod-threshold values during dark adaptation amongst different groups of subjects, viz
341 students of different institutions and 161 employees
The students and employees were of apparently
normal health and had no eye lesions*

THRESHOLD VALUES ON COMPLETE DARK ADAPTATION			FREQUENCY DISTRIBUTION OF THE DIFFERENT GROUPS OF SUBJECTS				
D R values	Millfoot candles	Log micro micro lambert	96 Hare School students	170 Hindu School students	75 Moti Seal Free School students	341 students (total)	161 employees
90	0 0023	3 3617	19	49	21	89	12
85	0 0035	3 5441	18	22	14	54	14
80	0 0051	3 7076	37	56	25	118	38
75	0 0077	3 8865	8	9	8	25	16
70	0 0114	4 0569	11	22	2	35	37
65	0 0170	4 2304			2	2	7
60	0 0252	4 4014	2	6	1	9	14
55	0 0375	4 5740		1	1	2	5
50	0 0557	4 7459		3		3	5
45	0 0830	4 9191		1		1	1
40	0 1240	5 0934	1	1	1	3	4
35	0 1840	5 2648					1
30	0 2740	5 4378					1
20	0 6040	5 7810					3
15	0 8980	5 9533					1
< 0	> 2 9600	6 4713					2

Amongst the employees there were some who had recovered recently from one ailment or the other. These cases and their D R values are given below. Further, the D R values of persons whose diet differs substantially from the average are also given below.

- (i) 1 subject recently suffering from intestinal troubles had D R = 50
- (ii) 1 subject had an attack of dysentery 6 months ago and of kala azar a little before the examination D R. = 40
- (iii) 1 subject recently suffering from malaria and chronic dysentery D R = 50
- (iv) 1 subject just previously suffering from malaria although taking a good diet D R. = 45
- (v) 1 just previously suffering from kala azar and spleen trouble D R = 20
- (vi) 1 previously suffering from kala azar but usually taking a good diet D R. = 60
- (vii) 4 subjects suffering from liver troubles D R = 20, 20, 20 and 70. First 3 taking a very poor diet, the last one a very good diet
- (viii) 3 cases taking purely a vegetarian diet D R = 40, 50 and 50, had little or no milk
- (ix) 1 case taking purely vegetarian diet but enough of milk D R = 70
- (x) 1 previously suffering from typhoid and also from chronic dysentery D R = 50

Discussion —It would be observed that amongst the 341 students (Table III) a large proportion (i.e. 77 per cent nearly) had the final threshold value of 80 D R or above, corresponding to 0.0051 m f c or even lower values, and that even amongst the poor students of the free school, the proportion having the above-mentioned final threshold value is not lower than 77 per cent. As regards the employees, only 40 per cent of the persons examined had D R of 80 and above but nearly 73 per cent showed a D R of 70 and above.

From Graph 5 which shows the frequency distribution with regard to final threshold values of 361 students and 161 employees of apparently normal health and with no distinct eye lesions, it is noticed that amongst the boys the distribution frequency is overwhelmingly maximal at $D R = 80$, but amongst the employees it shows two peaks, one at 70 and another at 80. In view of these considerations $D R = 80$ may be taken as normal for boys and $D R$ between 70 and 80 for adults.

In the footnote to Table III the $D R$ values are given of certain employees who had recently recovered from one or other ailment and of others whose diet was substantially different from the average diet of these persons. It is noticed that persons who suffered from spleen or liver troubles had the lowest $D R$, and then those who were living on a purely vegetarian diet without milk.

Effects of administration of large doses of vitamin A per diem to some of these deficient persons —Nearly 50 persons were selected for these experiments, but only 8 of them could be re-examined, as others did not turn up after the intake was over. The results of intake of vitamin A are given below in detail in Table IV.

It is evident that while there was a positive improvement in the light perception in all the cases, there was no marked improvement in Bitot's spots or pigmentation. It may be that they are either produced by multiple factors or that, being caused by prolonged vitamin A deficiency, they can be cured only by a prolonged intake of large doses of the vitamin.

EXPERIMENTS ON THE OPTIMUM REQUIREMENTS OF VITAMIN A

In assessing the vitamin A deficiency of persons the question naturally arises what is the optimum requirement of vitamin A of different persons. If we consider the optimum requirement of vitamin A as that amount which will confer on a person the maximum power of adaptation in the dark, it becomes necessary to find out what that power is, i.e. what is the highest value of $D R$ that can be obtained in a healthy person when he is saturated with vitamin A. It would then be necessary to find out what amount of daily intake of vitamin A can maintain a person at that condition of light perception in the dark, which is exhibited by a saturated person. Experiments were, therefore, performed to saturate persons by large doses of vitamin A and to note if their $D R$ values attain a steady state, then to ascertain if the $D R$ values at the steady state are the same or different with different persons and further to find out at what minimal dose this $D R$ value at the steady state is maintained.

The results of these experiments are given in Table V.

Assessment of Vitamin A Deficiency

TABLE IV
Showing the effects of administration of large doses of vitamin A daily to some deficient subjects

Number	Name	Age in years	Initial D R value	Eye complaints of the subject	Supplement of vitamin A in I U	D R values on different days after the ingestion of the supplement and the effect of the latter on the course of the disease
1	R B	25	40	Night blindness and Bitot's spot	10,000 I U daily	11th day 50, 18th day 65, 24th day 75, 39th day 80 The patient considerably improved in health, night blindness disappeared, but Bitot's spots not ameliorated to any noticeable degree
2	T C B	17	20	Night-blindness and liver troubles	"	9th day 36, 35th day 75 Night blindness much improved
3	K C J	34	45	Night-blindness	"	8th day 50, 25th day 70 Night-blindness much improved
4	H L B (one eyed)	17	20	Night blindness and xerosis	"	6th day 30, 17th day 50, 45th day 75—both defects much improved
5	Anrulla*	22	< 0	Night blindness, xerosis, pigmentation and Bitot's spot	"	13th day 10, 40th day 40 The first defect only slightly improved
6	Tukaram	22	50	Night blindness and pigmentation	"	15th day 75, 26th day 85, 53rd day 90 Night blindness considerably ameliorated
7	A B	7		Severe phrynoderma—mental disorder	"	Owing to dementia of the patient, dark-adaptation values could not be had Phrynoderma completely cured in 5 weeks' time
8	One baby	†		Severe keratomalacia	"	Completely cured in 4 weeks' time

* Initially, i.e. before administration of the vitamin A supplement, this subject could not see the test spot even at its maximum illumination

TABLE V
Showing the effects of continued ingestion of different doses of vitamin A on the final light threshold values in the dark

Number of subjects	Name of the subject	Age in years	Initial D R values	Supplement of vitamin A given at the beginning of the experiment	D R values on different days after the daily ingestion of varying amounts of the supplement or after it has been stopped
1	K C R	30	70	10,000 I U daily (plus usual diet)	7th day 74, 13th day 80, 17th day 85, 19th day 86, 24th day 90, 26th day 90, supplement of 5,000 I U daily 11th day 85 now supplement with vitamin A free diet*—ingested—in conjunction with vitamin A free diet*—8th day 90, 15th day 90, 20th day 90, 30th day 90
2	B C	22	90	10,000 I U (plus usual diet)	4th day 90, 11th day 90, 10th day 90, 35th day 90
3	P B	22	00	5,000 I U (plus vitamin A deficient diet)	3rd day 90, 15th day 90, 24th day 90, 37th day 90
4	B C (S)	30	70	10,000 I U (plus usual diet)	9th day 85, 17th day 90, 30th day 90, 40th day 90
5	N K D	34	80	5,000 I U (plus vitamin A deficient diet)	12th day 90, 25th day 90, 35th day 90
6	R P	27	80	5,000 I U (plus usual diet)	8th day 85, 13th day 90, 18th day 90, 25th day 90, supplement stopped—5th day 90, 8th day 90, 13th day 85, 23rd day 80, diet from the next day was practically devoid of vitamin A but supplemented with 4,000 I U vitamin A daily fed orally—11th day 80, 26th day 80, supplement stopped but continued on a deficient diet—8th day 75, 5th day 75, 12th day 80 supplement stopped—8th day 70, 15th day 70, from the next day kept on a practically vitamin A free diet supplemented with 3,000 I U of vitamin A daily fed orally—5th day 70, 10th day 70, 20th day 70—supplement stopped but continued to take the deficient diet—5th day 65, 12th day 60
7	S G	18	70	10,000 I U (plus usual diet)	

* The vitamin A deficient diets consisted of the usual food materials strictly excluding the vitamin A rich substances, e.g. milk, butter, ghee, eggs, leafy vegetables, carrots, oranges, tomatoes, papaya, etc

Discussion—It is obvious from these experiments that a steady state with regard to adaptation is reached during saturation and that this steady state is the same, viz a D R value of 90 for all persons. This value cannot be exceeded even after prolonged intake of 10,000 I U of vitamin A. On examining Tables I, II and III, it will be observed that the D R value of 90 was never exceeded by any person between the ages of 6 and 60.

Further analysis of Table V shows that the D R of 90 is maintained with a daily dose of 5,000 I U of vitamin A (*cf* experiments with K C R, P B and N K D). Accordingly, 5,000 I U of vitamin A *per diem* may be considered as the optimum requirements of vitamin A for adults. Booher *et al* (1940) also considers the optimum requirements of vitamin A for industrial workers to be 5,000 to 6,000 I U *per diem*.

Experiments with R P and S G (Table V) reveal that a daily dose of 4,000 I U of vitamin A was required to maintain the D R value at 80, and a dose of 3,000 I U to maintain the D R at 70. Since a very large proportion (77 per cent) of the healthy boys gave a dial reading of 80 or above (*vide* Graph 5), 4,000 I U of vitamin A may be accepted to be the normal or minimum requirements for boys per day. Again since majority (73 per cent) of the employees had a D R of 70 or above (*vide* Graph 5), 3,000 I U of vitamin A may be accepted to be the normal or minimum requirements for adults per day.

SUMMARY

1 Thirty-nine persons were examined with a biophotometer according to Jeans' method for the assessment of the vitamin A content in their bodies. It was found that borderline cases cannot be detected by this method and that there is no correspondence in several cases between the dietary intake of vitamin A and the light-threshold value obtained by this instrument.

2 In consequence of this discrepancy both the instrument and the technique were modified in accordance with the suggestions of Hecht. Fifty-four apparently normal persons were then examined according to the method of Hecht by the modified instrument. A fair amount of correspondence was observed in the case of a majority of subjects between the vitamin A intake and the light-threshold value at 30 minutes after the extinction of the bleaching light. But there were certain cases in which there was no correlation between the observed D R values and the dietary intake. This absence of correlation is found to be due to certain inherent defects of the method, and the means of eliminating these have been discussed in the paper.

The great disadvantage of Hecht's method is the time (*viz* an hour) required for the examination of each person. Accordingly, it cannot be applied for a large scale determination of the vitamin A status of persons.

3 In view of this great disadvantage of Hecht's method, a modified method has been suggested by the authors, the rationale of which has been discussed in the text. According to this modified method a batch of 12 persons are simultaneously

examined in the dark, without any preliminary dark adaptation and exposure of their eyes to a strong bleaching light, and their final light-threshold values in the dark are ascertained. These values give the vitamin A status of the subjects.

Three hundred and ninety-one apparently normal school boys from families of different economic status and 161 employees of moderate income were examined by this method.

Nearly 77 per cent of the boys were found to have a high D R value of 80 or above on complete dark adaptation, indicating that the vitamin A content in their bodies was normal. Amongst the employees although only 40 per cent had reached this high D R value, nearly 73 per cent did actually satisfy the minimal value for adults, i.e. their vitamin A status may not be regarded as deficient. After prolonged administration of large doses of vitamin A to some patients, their D R values were invariably raised, but symptoms, such as pigmentation of the conjunctiva or Bitot's spots were not cured. The apparent absence of any effect of ingestion of vitamin A in large doses, on Bitot's spots and pigmentation, might be due either to the fact that they are caused by multiple factors or that a much more prolonged intake is necessary to remove these symptoms.

4. The optimum requirements of vitamin A for an individual are concluded to be 5,000 I U daily, while the minimum requirements for the boys and adults 4,000 and 3,000 I U respectively.

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OBSERVATIONS ON THE PHYSICAL DEVELOPMENT OF PUNJABI BOYS

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SUBJECT OF THIS STUDY.

A good many surveys of physical development in Indian children have been carried out (Aykroyd and Rajagopal, 1936, Aykroyd, Madhava and Rajagopal, 1938, Chatterjee, 1938, Mitra, 1939, Narindra Singh, 1939, Shourie, 1939, Wilson, D C, 1939, Wilson, H E C and Mitra, 1938). However, the population of India is so heterogeneous that it may be of value to add to the surveys already carried out one from the Punjab.

We studied the weight and height of about 1,500 Punjabi boys from 5 to 23 years of age. Next various body measurements were recorded, such as length of legs, chest girth, etc., and compared with those of other groups. From these data we have calculated certain proportions and indices.

Students in a Mission High School and in Gordon College in Rawalpindi, and also in a Mission High School in Sialkot, were examined. This was a yearly routine medical examination. Of these groups the college students are under the most comprehensive medical supervision.

RESULTS

A Absolute body measurements—Table I shows average values for various measurements in the different age groups. The number of subjects is given in brackets.

(1) Column 1 in Table I shows the weight averages from 5 to 23 years. In Graph 1 these are compared with those of Bengali, Kashmiri, Assamese and

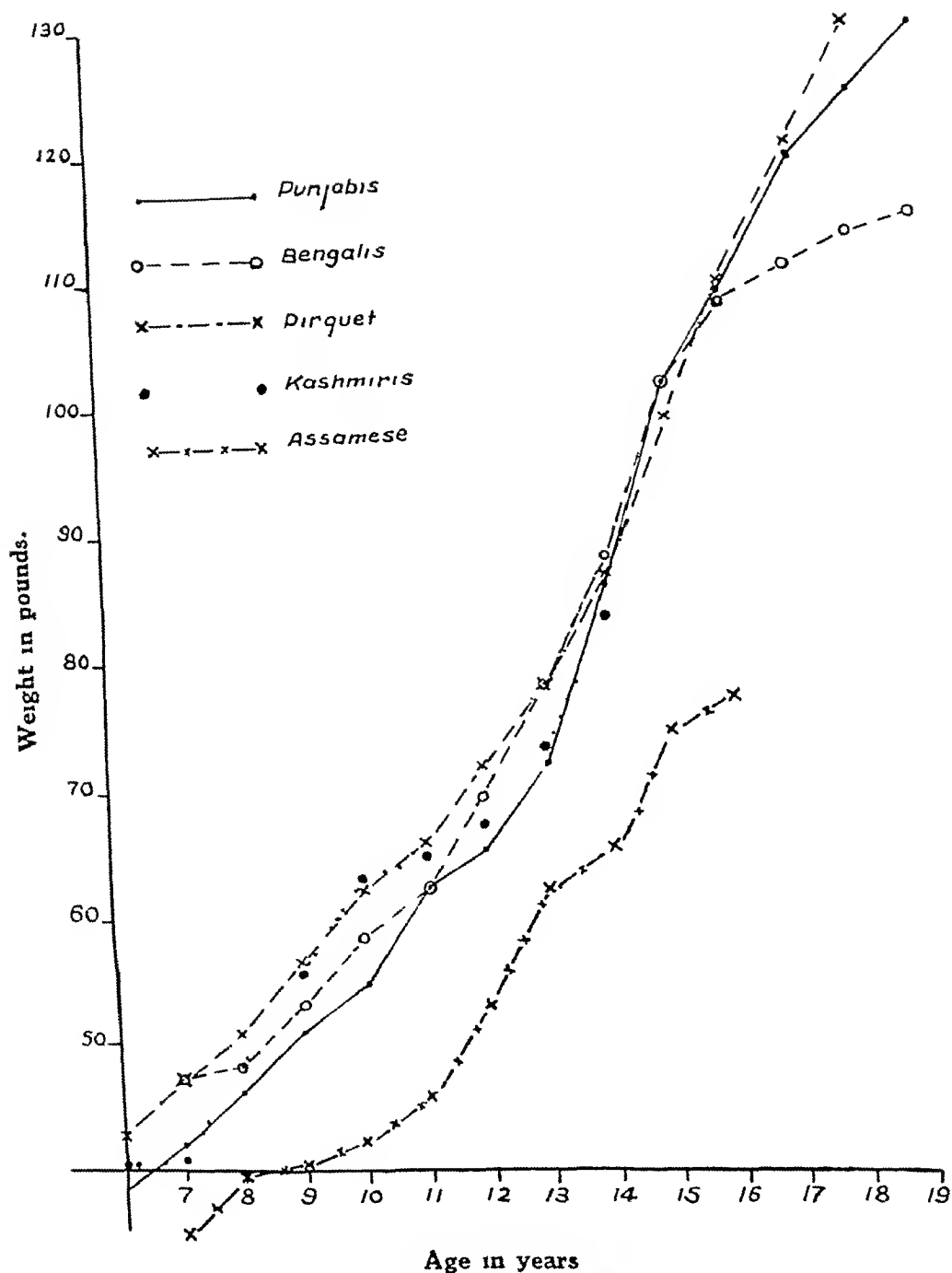
TABLE I.

Average values for various measurements of Punjab boys

Age	1	2	3	4	5	6	7	8	9	10
	Weight Lb	Height, Ft Inches	Chest girth expiration Inches	Chest girth inspiration Inches	Sagittal chest diameter expiration Inches	Sagittal chest diameter inspiration Inches	Abdominal girth Inches	Biceps Inches	Calf Inches	Length of leg Inches
5	34.6 (18)	3-5.4 (18)	20.5 (18)		5.1 (17)		20.2 (16)			20.0 (16)
6	38.7 (75)	3-7.9 (75)	21.2 (75)		5.3 (75)		20.3 (68)			21.3 (69)
7	41.8 (74)	3-10.0 (74)	21.7 (74)		5.3 (74)		20.0 (65)			22.0 (66)
8	46.4 (60)	3-11.8 (60)	22.0 (60)		5.4 (60)		20.8 (60)			24.0 (60)
9	50.0 (95)	4-0.8 (95)	22.7 (95)		5.5 (95)		21.2 (94)			24.7 (94)
10	55.0 (111)	4-3.6 (110)	23.3 (111)		5.7 (91)		21.6 (111)			26.5 (111)
11	62.3 (117)	4-5.9 (117)	24.0 (120)		5.8 (95)		21.8 (119)			27.7 (118)
12	65.2 (129)	4-6.6 (130)	24.6 (127)		5.9 (85)		22.2 (129)			28.6 (128)
13	72.1 (86)	4-9.0 (85)	24.8 (86)	27.6 (23)	6.03 (72)	6.0 (3)	23.0 (85)	8.7 (3)	11.7 (3)	29.8 (84)
14	85.7 (85)	5-0.4 (86)	26.2 (85)	29.4 (45)	6.4 (76)	7.5 (13)	24.2 (84)	9.5 (17)	11.9 (17)	31.5 (67)
15	102.0 (133)	5-3.3 (133)	27.6 (133)	30.7 (118)	6.7 (119)	7.8 (75)	25.2 (131)	9.6 (87)	12.1 (88)	32.6 (45)
16	110.0 (188)	5-4.4 (187)	28.3 (188)	31.2 (182)	7.0 (156)	8.0 (128)	25.9 (180)	9.9 (150)	12.3 (100)	34.1 (27)
17	119.0 (150)	5-5.2 (150)	29.6 (150)	32.4 (150)	7.2 (103)	8.1 (103)	26.8 (140)	10.2 (150)	12.6 (150)	
18	124.0 (128)	5-6.3 (128)	30.2 (126)	33.1 (127)	7.4 (82)	8.4 (82)	27.4 (124)	10.5 (128)	12.7 (128)	
19	130.0 (101)	5-6.5 (101)	30.6 (101)	33.3 (101)	7.7 (61)	8.8 (61)	27.9 (98)	11.1 (97)	12.8 (100)	
20	126.0 (59)	5-7.3 (59)	29.8 (58)	33.0 (58)	7.4 (33)	8.4 (33)	27.9 (55)	10.9 (55)	12.7 (55)	
21	128.5 (29)	5-6.9 (29)	30.8 (28)	33.5 (28)	7.4 (11)	8.5 (11)	28.1 (28)	10.9 (28)	12.8 (28)	
22	132.6 (14)	5-7.3 (14)	31.6 (14)	34.6 (14)	7.8 (9)	9.1 (9)	28.8 (14)	11.4 (14)	13.0 (13)	
23	128.5 (9)	5-7.3 (9)	30.8 (9)	34.4 (9)	7.8 (4)	8.9 (6)	28.4 (9)	10.9 (9)	12.9 (9)	

Figures in brackets indicate number of subjects

GRAPH 1



Weight age averages of boys from the Punjab, Bengal, Europe (after Pirquet), Kashmir and Assam

Central European children (Chatterjee, *loc cit*, Wilson, D C, *loc cit*, Mitra, *loc cit*, Pirquet after Brock, 1930) The Bengal, Kashmir and European figures are slightly higher than those from the Punjab The differences are not considerable up to the age of 16, this coincides with previous experience The differentiation of development seems to take place only after puberty The graph shows that the weight of Punjabis and Europeans continues to increase steadily after 16, while the Bengal curve flattens out We have given in the diagram also the average weight of children in Assamese tea-estates as an example of a badly fed population The curves from Orissa (Narindra Singh, *loc cit*), rural Bengal (Mitra, *loc cit*), Marwari families from Calcutta (Mitra, *loc cit*), Delhi province (Shourie, *loc cit*) and South India (after Narindra Singh, *loc cit*) fall between those of Punjabi and Assamese children It is noteworthy that Chatterjee's values, based on high school and college students in Bengal, are nowhere reached by those of Wilson, H E C and Mitra (*loc cit*)

(2) Column 2 of the table gives height averages In Graph 2 these are compared with figures from the other groups Up to the age of 15, Bengalis (after Chatterjee, *loc cit*) are slightly taller than Punjabi boys After this age is reached the Bengali curve flattens out, and the Punjabi curve 'overtakes' it at 18 So does the curve for European children At 17 to 18 years, the Bengali curve becomes quite flat, while the European curve is still ascending steeply The Punjabi curve shows a medium slope The Assamese values for height, like those for weight, are low in all age groups The figures from other districts again lie between those given by the Punjabi and Assamese children, though nearer to those of the Punjabis

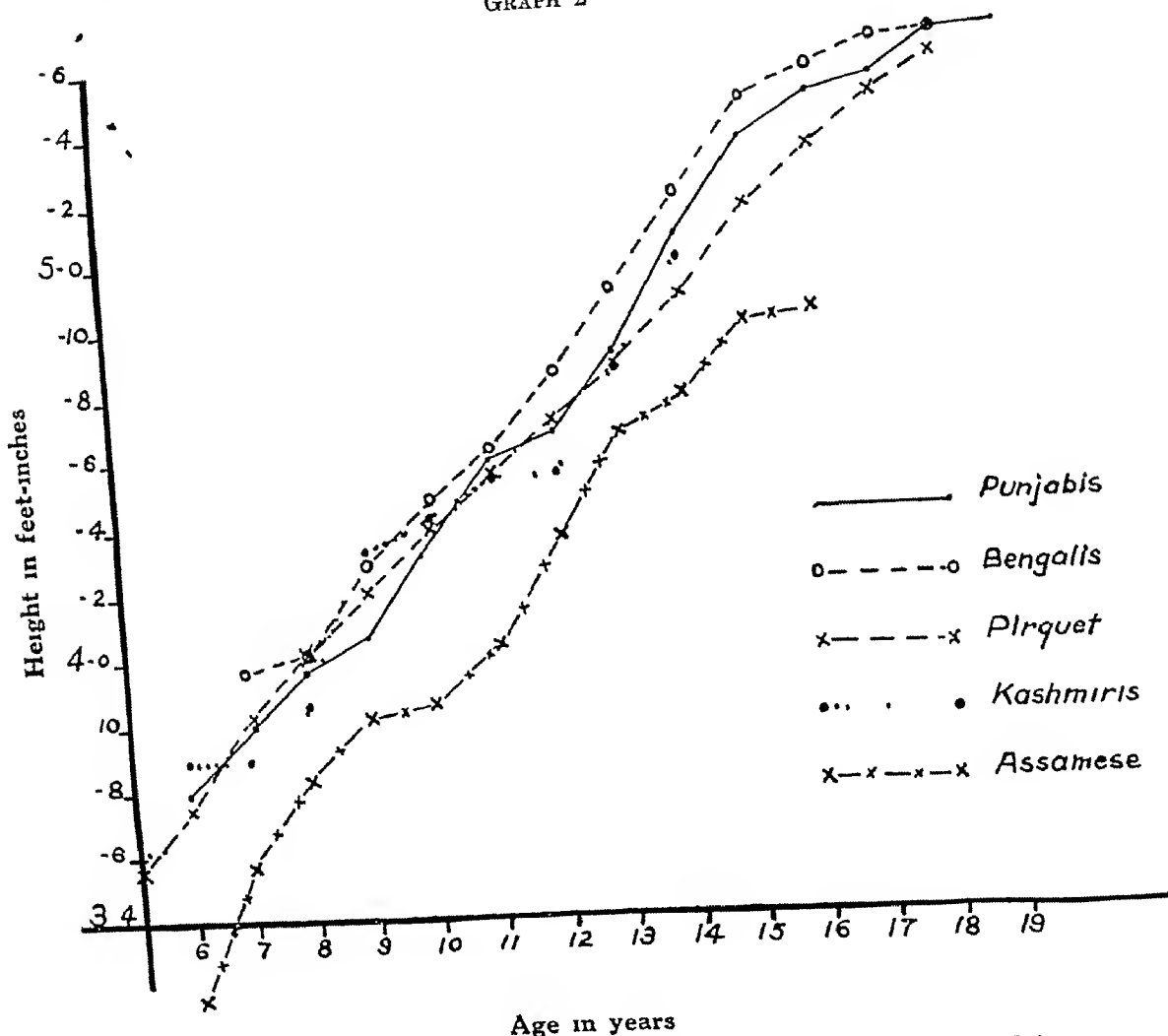
There is a slowing of growth in Punjabis and Marwaris (vegetarian, town-dwelling middle class people from Calcutta, non-Bengalis, after Mitra) between 11 and 12 years In Kashmiris (Wilson, D C, *loc cit*) there is almost a cessation of growth at the same period In the children from Delhi province (Shourie, *loc cit*) a decreased rate of growth is found between 10 and 11, in Assamese between 9 and 10 years Further investigation is necessary to discover whether this phenomenon is due to too small a number of observed cases or if an actual retardation of growth rate takes place

Brock (*loc cit*) shows that differences in height at birth in different races are inconsiderable and that it is after puberty that differentiation in stature takes place In general early puberty makes for an earlier conclusion of growth and therefore for smaller size Friedenthal (quoted by Brock, *loc cit*) points out that between the age of 9 and 14½ years Japanese are taller than European children, at 20, however, the Japanese are about 6 per cent smaller than Europeans Weissenberg (quoted by Brock, *loc cit*) has suggested that the later sexual maturity occurs, the greater the final stature attained

(3) Columns 3 and 4 of the table show chest girth measured at expiration and inspiration We measured the circumference horizontally, immediately below the lower angle of the scapulæ The average values for the expanded chest coincide between 18 and 19 years with Chatterjee's (*loc cit*) values It is noteworthy that at the beginning of puberty, i.e. in Punjabis at the age of 13 years, the chest

circumference grows much more rapidly than before Bengalis show this increase about 2 years earlier Perhaps Weissenberg's law for length can be applied to other dimensions We can see that the curve of Bengalis flattens out at 16 years, whereas in the case of Punjabis chest circumference continues to increase between

GRAPH 2

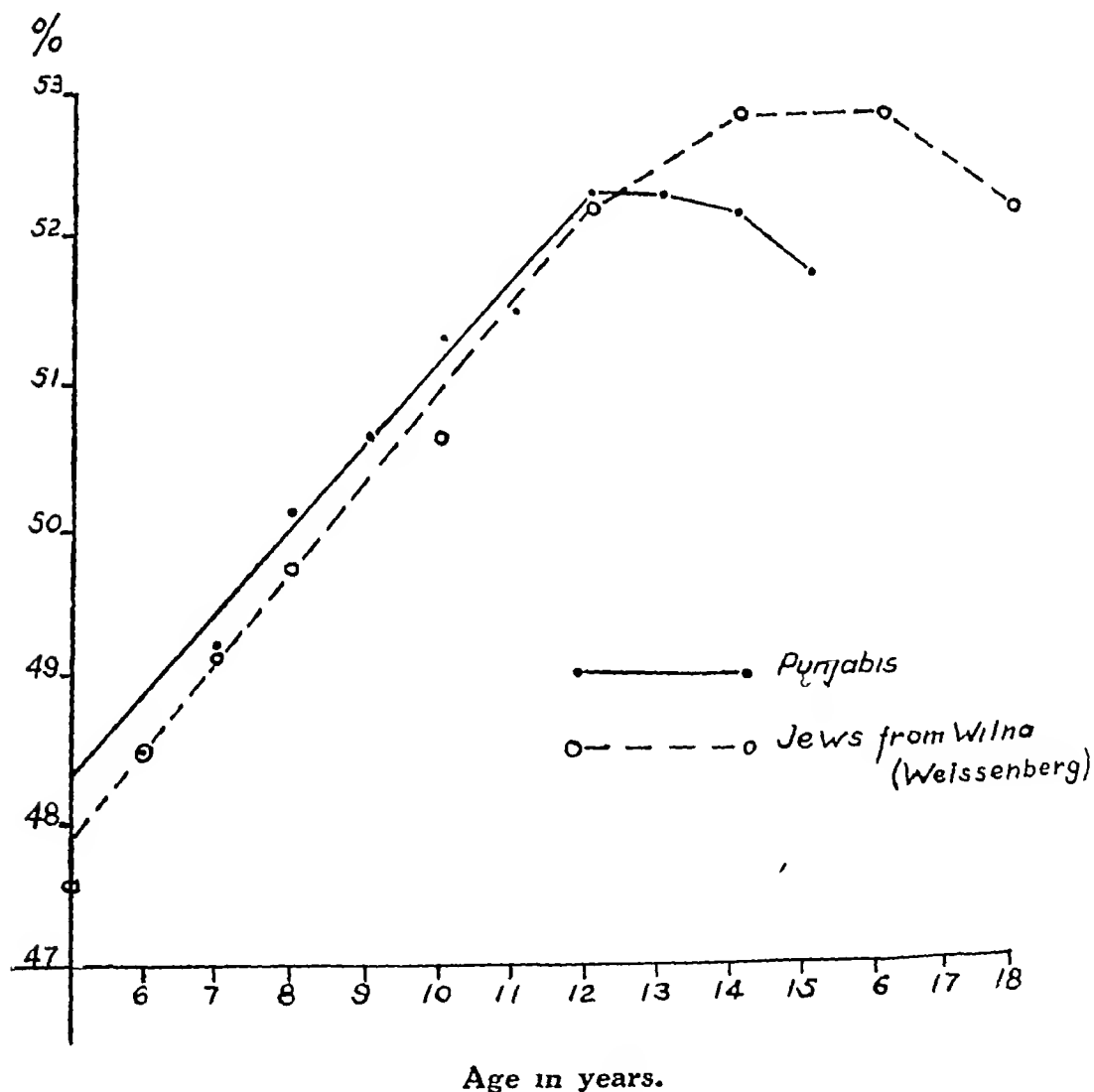


Height-age averages of boys from the Punjab Bengal, Europe (after Pirquet), Kashmir and Assam

17 and 18 years That too might indicate that Bengalis have an earlier puberty as we already discussed in paras (1) and (2) The chest circumference of Punjabis increases between 6 and 13 years by 0.5 inch per year, and between 13 and 18 years by 1.08 inch

(4) Columns 5 and 6 of the table show the sagittal chest diameter in expiration and inspiration. The diameter was measured with the help of a wooden compass with parallel branches at the level of the 9th rib. The rate of increase in these measurements becomes more rapid at the age of 13 years, and is more pronounced after 13 years than the increase of the circumference values of column 3. This

GRAPH 3

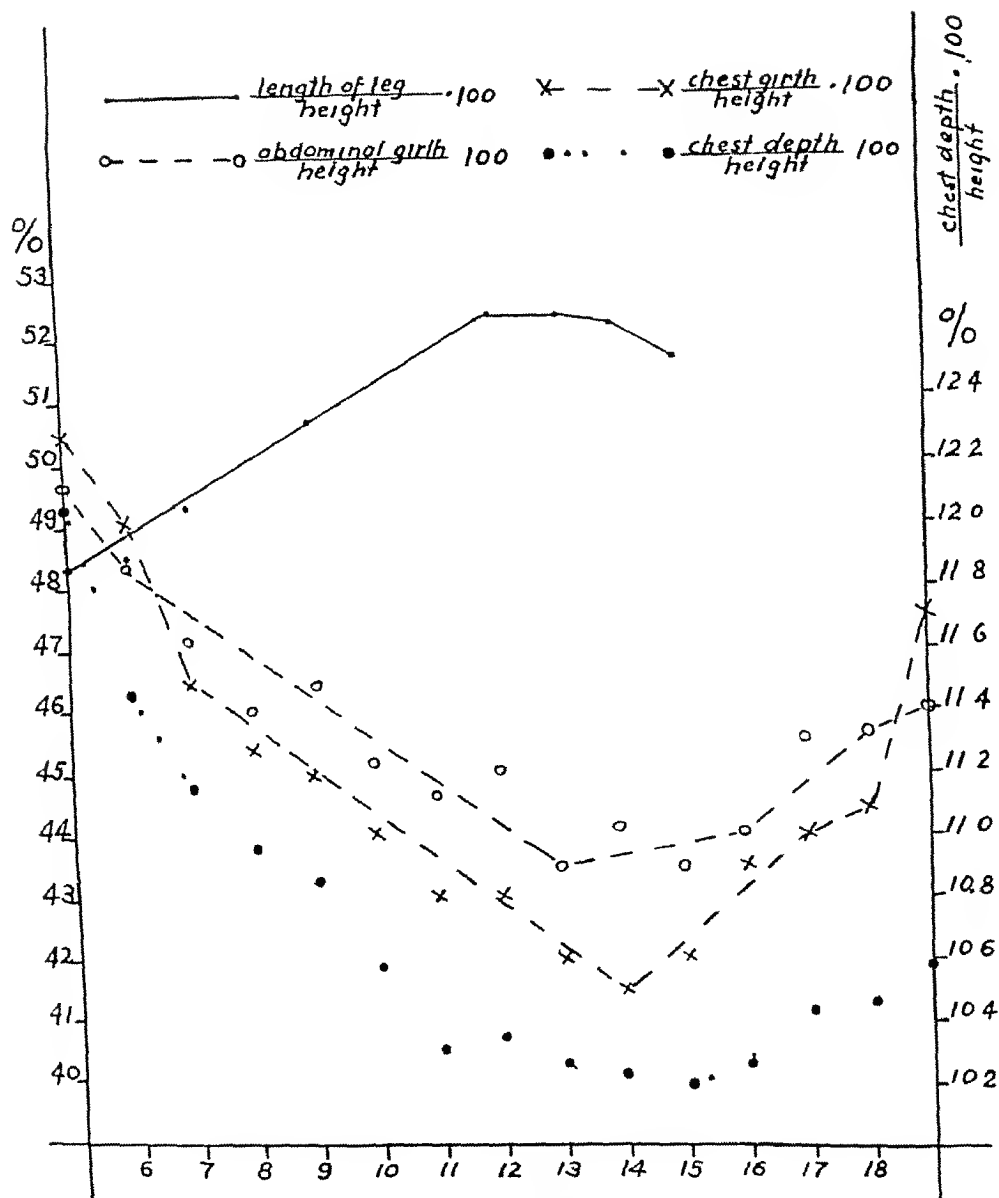


Length of leg
height 100, comparing Punjabis and Jews from Wilna

means that after the beginning of puberty the sagittal diameter i.e. the depth of chest, increases more rapidly than the transversal diameter.

(5) Column 7 in the table gives the abdominal circumference. These values show a behaviour similar to that of the values of chest circumference.

GRAPH 4



Age in years.

Length of leg abdominal girth chest girth and chest depth (sagittal chest diameter)
in per cent of height, of Punjabi boys

(6) Columns 8, 9 and 10 give the biceps and calf circumference and the length of leg. Biceps and calf were measured at the point of their greatest circumference, the length of legs from the tip of the trochanter major to the ground. The leg measurements are discussed later with the analysis of proportions.

B Proportions—The general structure of man (see Brock, *loc cit*), if followed up from foetal development to maturity, shows extensive changes. The changing of the proportions of various parts of the body in relation to each other and the total size are of considerable interest. There is the possibility that these changes of proportions are dependent upon certain hereditary or environmental factors and vary in different groups of people.

In Table II, columns 1 to 4, we give the proportions of leg, chest girth, chest diameter and abdominal girth to total body size respectively. Graphs 3 and 4 show the proportions graphically. Graph 3 compares the leg/height proportions of Punjabis with the values given by Weissenberg for Jews from Wilna.

TABLE II
Various proportions (in percentage of height)

Age	1	2	3	4
	Leg	Chest girth	Sagittal chest diameter	Abdominal girth
5	48.3	40.6	12.3	40.2
6	48.5	48.8	12.0	46.3
7	49.2	47.2	11.5	44.8
8	50.2	46.0	11.3	43.8
9	50.6	46.5	11.2	43.4
10	51.4	45.2	11.0	41.9
11	51.5	44.6	10.8	40.5
12	52.3	45.1	10.8	40.7
13	52.3	43.5	10.6	40.3
14	52.2	44.1	10.5	40.1
15	51.2	43.6	10.6	39.9
16		44.0	10.9	40.3
17		45.5	11.0	41.1
18		45.6	11.1	41.3
19		46.1	11.7	42.0

The study of the graphical pictures of these four proportions (Graph 4) shows two diametrically opposed types. The leg/height proportion ascends from the 5th to the 12th year in a more or less straight line. That means that the length of leg and total body size grow at uniform rate. After 12 years the curve turns sharply, indicating that the growth of the legs becomes comparatively slower. In this period increase in length of the body is due mainly to growth of the trunk.

The curves of Punjabis and Jews from Wilna (Graph 3) show an analogous behaviour. In the case of the latter the turn in the curve comes at 14 instead of at 12 years as in the case of the former.

The three other proportions, i.e. chest girth, chest diameter and abdominal girth/height, descend during the period (from 5 to 12 years) in which the leg/height proportion increases. Between 12 and 15 years, they ascend, this is in the period in which the leg/height proportion decreases. This can be explained by the fact that the leg/height proportion is one comparing two measurements of extensions which are parallel to each other and to the body axis. The other three proportions bring into relation longitudinal, sagittal and transversal extensions. The descending parts of these three curves relate to a period in which growth in length predominates, while the ascending parts are an expression of the filling up of the body.

C Indices—There have been attempts to find formulæ which can be used to determine whether an individual possesses the weight appropriate to his size or vice versa. The body measurements employed in these formulæ have to be very simple, in order to make them applicable in practice. Among the best known indices are those of Quetelet $\left(\frac{P}{L}, \frac{P}{L^2}, \frac{P^2}{L^5} \right)$, Rohrer $\left(\frac{P}{L^3} 100 \right)$,—here stands P for weight and L for length—and Borchardt

$$\frac{(\text{Weight minus chest girth}) \text{ multiplied by length}}{240}$$

There is also the arm-chest-hip index mainly employed for the detection of malnutrition and discussed by Aykroyd, Madhava and Rajagopal (*loc cit*). Not all of these are mathematically correct as they bring into relation factors of different mathematical dimensions, e.g. weight to length or weight to surface. We propose to limit our discussion to the mathematically correct Rohrer index, i.e. weight over the cube of length.

Table III shows the Rohrer index for our Punjabi boys compared with that calculated from the Pirquet tables after Brock (*loc cit*). A first glance shows that the index is not a constant. On the contrary, it falls from relatively high values in the early school age to a minimum between 12 and 14 years and later ascends again. This means that the relation of weight to length does not remain the same during the development of the child. From 5 to 12 years the increase of length is greater than the increase of weight. Later on this ratio is reversed. The well-known adolescent type, rather lean and with long legs, approaches more and more to the

adult type which is characterized by better muscular development, greater fat accumulation and predominance of length of trunk

TABLE III.

Rohrer indices.

Age	Punjabis	Europeans after Pirquet
5	4 88	4 88
6	4 48	4 27
7	4 31	4 13
8	4 23	4 00
9	4 37	3 88
10	3 99	3 84
11	4 00	3 75
12	4 00	3 72
13	3 90	3 68
14	3 90	3 62
15	4 02	3 71
16	4 13	3 78
17	4 32	3 81
18	4 25	3 88
19	4 43	3 88

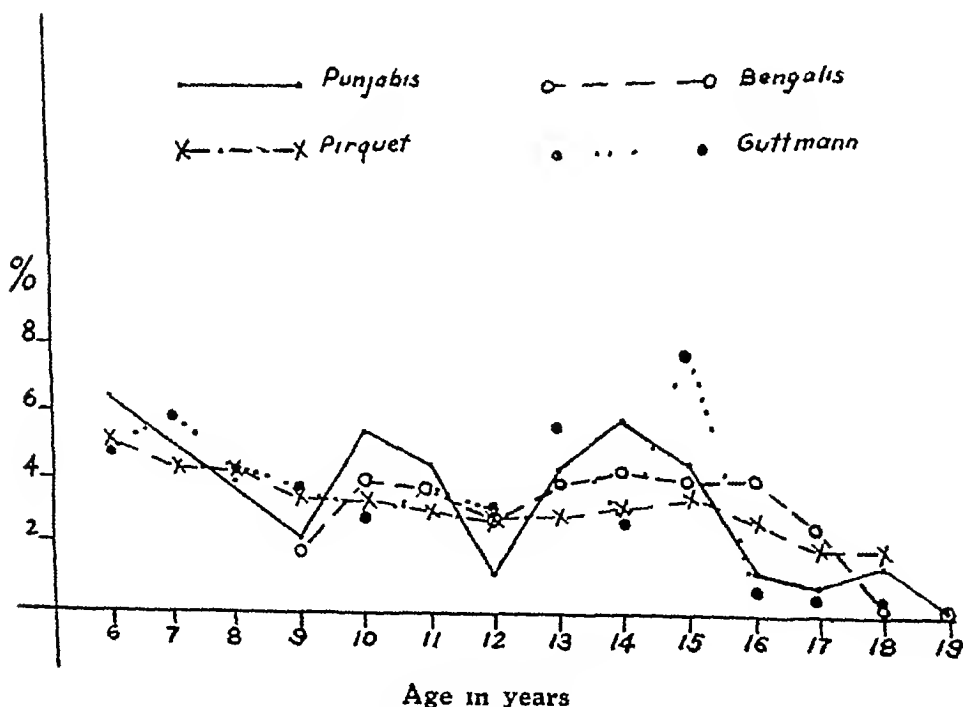
The importance of the indices in practice is comparatively small, as they do not represent biological constants, i.e. figures which are the same for all age groups. Their use therefore has been more or less abandoned for clinical purposes, but their importance for biometric investigations remains.

It is interesting to compare the values of the Rohrer index with the curves of the proportions as given in Table II and Graph 4. The shape of the Rohrer curves would be almost identical with that of the index curves. The meaning of this similarity is as follows: the curves of proportions are an expression of the fullness of the body just as much as the curves of the indices. The proportions reflect the ratio of square roots of body cross sections to size. They therefore do for certain parts of the body what the Rohrer index achieves in a more comprehensive way for the whole body (weight over cube of length resp. cubic root of weight over length).

D Increment in height and weight—The growth coefficient or increment in height and weight will now be considered. The rate of growth falls from birth to about the age of 20, when it comes to a standstill. This decrease of intensity of growth however does not run in a straight line. At the time of puberty there is a marked increase in the rapidity of growth. The puberty phase of growth is of great importance because upon it depend the height differences of the two sexes. The differentiation of racial types as far as dimensions are concerned occurs also in this period [see para A (2)]

Graph 5 gives the height increment of Punjabis, Bengalis and European children after Pirquet, and the values obtained by Guttman (Brock, *loc cit*)

GRAPH 5



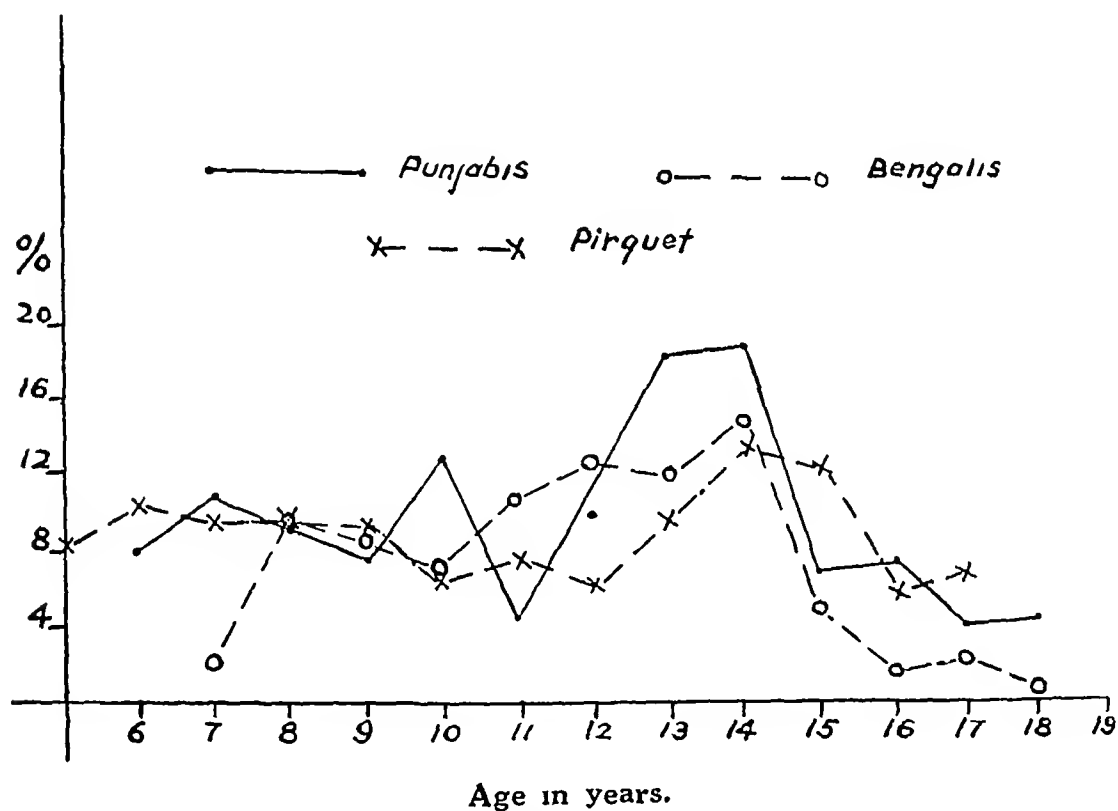
Height increments of Punjabis, Bengalis, Pirquet and Guttman children (Europeans)

In Pirquet's curve the maximum of growth intensity occurs at 15 years, as it does in Guttman's curve. The Punjabi and Bengali curves show the maximum distinctly at 14 years. This re-affirms the generally accepted view of the influence of puberty upon the growth coefficient.

Both Punjabis and Bengalis show another period of growth acceleration at 10 years with a minimum at 12 years.

The weight increment is shown in Graph 6. We have again compared Punjabi, Bengali and European children. All three curves show between the 12th and the 15th year an increase of weight increments. It is interesting to note that the increase begins with the Bengalis at 11, with the Punjabis at 12 and with the Europeans at 13 years. The maximum is in all cases at 15 years. But at 18 years there are again differences to be observed. Europeans still maintain a weight increment of 7 per cent, Punjabis of 4 per cent and Bengalis less than 3 per cent, in contrast to what occurs at the beginning of the acceleration.

GRAPH 6



Weight increments of Punjabis, Bengalis and Pirquet children

SUMMARY

Data were collected about the physical development of Punjabi boys. Their various body measurements were compared with those of other Indian and European groups. From the figures collected, certain biometrical proportions, indices, and rates of growth were calculated and discussed in comparison with those of other groups.

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BLOOD UREA CLEARANCE IN NORMAL INDIANS

A STUDY BASED ON THE EXAMINATION OF 110 NORMAL INDIAN MEN

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BLOOD UREA clearance test introduced by Möller, McIntosh and van Slyke (1929) has come into wide use in recent years as the most sensitive test for estimating renal efficiency in kidney disease. Gershenfeld (1933) remarks that it is 'capable of estimating renal insufficiency much earlier and even the degree of renal functional damage, being a better indicator of the extent of anatomic renal changes (both glomerular and vascular), than is obtainable by other kidney functional tests'.

Use of this test in the routine diagnostic work carried out in this Institute almost always gave low results showing extremely deficient kidney function. These laboratory findings did not, however, seem to tally with the clinical findings. The examination of a few normal individuals also yielded low values. This brought into question the validity of the use of American standards for measuring the renal function in the case of Indian subjects. Blood urea clearance is a relationship between the urea in blood and that excreted in urine in a given space of time, and since it had been found by the author (Gokhale, 1939) that the blood urea content of normal Indians had the same value (9 mg to 15 mg N per 100 c.c.) as in normal Americans, the low clearance values could only be due to lower excretion of urea in urine by Indians. That it was probably so, was indicated by the low nitrogen excretion in urine observed in a previous study (Gokhale, *loc cit*).

To clear this issue, 24-hour collections of urine of 47 normal Indian subjects were analysed and their volume, total nitrogen and urea nitrogen contents determined (method Folin, 1934). The results are summarized in Table I —

TABLE I

Total nitrogen and urea nitrogen excretion in 24-hour collection of urine of 47 normal Indian subjects

	Average	Range
Total nitrogen	6.09 g	3.62 g to 9.84 g
Urea nitrogen	4.65 g	2.74 g to 7.54 g
Volume	1,279 c c	480 c c to 2,670 c c

The average values obtained for total urinary nitrogen and urea nitrogen, viz 6.09 g and 4.65 g respectively, are definitely much lower than the corresponding average values for those of Americans. Total nitrogen 12 g to 18 g, urea nitrogen 10 g to 16 g (Hawk and Bergeim, 1938), urea nitrogen 11 g (MacKay and MacKay, 1927). This is in spite of the fact that the average volume of urine excreted in 24 hours is about the same in both the Indian and American subjects (1,200 c c to 1,300 c c). The average blood urea nitrogen content of normal Indian subjects is about 12 mg per 100 c c of blood with a range of 9 mg to 15 mg per 100 c c (Gokhale, *loc cit*) (cf also Tables II and III herein). The average blood urea nitrogen content for normal American and European subjects is also about 12 mg per 100 c c of blood with a range of 9 mg to 15 mg per 100 c c (Fohn and Svedberg, 1930, Berghund, 1922, MacKay and MacKay, *loc cit*, Priestley and Hindmarsh, 1924, quoted by Peters and van Slyke, 1931). Thus, while the average blood urea nitrogen content is the same in both the American and Indian subjects, the average urinary urea nitrogen excretion is very different. The reason for these differences lies in the difference of the diets of the two. The diet of the Indians generally contains very much less proteins than that of the Americans or Europeans and a diet poor in proteins will give a lower urinary nitrogen excretion but will not lower the urea nitrogen content of blood (Fohn, 1917). Only if protein is almost entirely cut out of the diet, will it lower the urea nitrogen in blood (Smith, 1926).

Thus, in view of the findings referred to above, that while the average blood urea nitrogen content is the same in both the American and the Indian subjects, the average urinary urea nitrogen is less, it is evident that the blood urea clearance value obtained for American normal subjects would not hold good for evaluating the kidney function of Indian subjects. Therefore, urea clearance value must be worked out for normal Indian subjects to establish a standard for use in blood urea clearance test in diseased kidney conditions. The present study was undertaken to provide this standard.

SUBJECTS EXAMINED

One hundred and ten normal Indian males were examined. The subjects were healthy young men mostly between 18 and 30 years of age. Most of them were from the province of Bombay. Fifty-eight of the subjects were vegetarians, while the remaining were on a mixed diet. There was no real marked difference

between the two diets (Sokhey *et al*, 1937, Gokhale, *loc cit*) Both groups subsisted more or less on the same sort of diet Both consumed milk, those on mixed diet ate in addition a little mutton now and then The average age of the subjects was 26 years Height and weight averaged 5 feet 4½ inches and 122.6 lb respectively giving an average body-surface area of 1.60 square metres

METHOD

The technique followed for the blood urea clearance test was the same as that described by Möller *et al* (*loc cit*) The subjects did not have any food, not even tea or coffee, after the usual meal on the night preceding the morning of the test Urine was completely voided just before the test was begun and a measured volume of water, viz 400 c.c. was given to the subject to drink and the time noted The subject remained quiet during the ensuing test period of two hours Urine was collected at the end of the first and the second hour, the volumes measured and urea nitrogen content estimated (Folin, 1934) About 50 minutes after the test began, a sample of 3 c.c. of blood was drawn from the cubital vein and collected in a small Erlenmeyer flask containing neutral potassium oxalate G. R. Merck (2 mg per c.c. of blood) Urea nitrogen of the blood was estimated by Folin's (1934) method

Möller *et al* (*loc cit*) have expressed blood urea clearance as a relation between urea concentration in urine (U), volume of urine excreted per minute (V) and urea concentration in blood (B), i.e. the volume of blood cleared of urea per minute In their study they have shown that when the volume of urine excreted per minute is more than 2 c.c. the clearance which they call 'maximum clearance', C_m , is expressed by the formula $\frac{UV}{B}$, and when the volume of urine excreted per minute is less than 2 c.c. the clearance called 'standard clearance', C_s , is expressed by the formula $\frac{U\sqrt{V}}{B}$

The results obtained in the present study are given in Tables II and III, 'maximum clearance' values are given in Table II and the 'standard clearance' values are given in Table III In the diet column, V stands for vegetarian diet and M for mixed diet —

TABLE II

'Maximum clearance' values in 37 normal young Indians

Number	Age in years	Diet	Surface area square metres	B Blood urea nitrogen mg per 100 c.c.	V Volume of urine c.c. per minute	U Urea nitrogen in urine mg per 100 c.c.	Maximum clearance, $\frac{UV}{B}$ c.c.
1	20	M	1.41	11.96	2.05	226	38.74
2	20	V	1.37	11.48	2.78	170	41.17

Blood Urea Clearance in Normal Indians

TABLE II—contd

Number	Age in years	Diet	Surface area square metres	B Blood urea nitrogen mg per 100 c c	V Volume of urine c c per minute	U Urea nitrogen in urine mg per 100 c c	Maximum clearance, $\frac{UV}{B}$ c c
						132	30 59
3	21	M	1 80	11 30	2 66	267	46 98
4	22	V	1 68	13 64	2 40	131	28 59
5	22	V	1 47	13 15	2 87	184	39 65
6	23	M	1 71	12 53	2 70	161	34 65
7	23	V	1 56	11 06	2 38	155	30 93
8	24	V	1 54	13 18	2 63	190	38 87
9	24	M	1 56	13 59	2 78	125	30 68
10	24	ML	1 64	10 08	3 20	165	39 73
11	24	M	1 46	12 75	3 07	212	52 79
12	24	V	1 44	12 57	3 13	137	37 38
13	24	V	1 43	12 90	3 52	205	42 21
14	24	M	1 52	13 84	2 85	169	40 65
15	24	V	1 68	11 30	3 32	83	32 83
16	24	M	1 55	10 87	4 30	83	37 12
17	24	M	1 55	9 86	4 41	224	40 45
18	25	V	1 46	13 18	2 38	120	44 45
19	25	M	1 51	10 69	3 96	195	33 45
20	25	V	1 81	13 70	2 35	205	35 96
21	26	M	1 49	13 51	2 37	219	44 22
22	27	M	1 57	14 71	2 97	181	45 59
23	27	ML	1 63	10 92	2 75	153	41 56
24	27	V	1 38	9 13	2 48	220	38 67
25	27	V	1 39	13 71	2 41	199	46 30
26	28	V	1 35	11 82	2 75	209	41 62
27	28	M	1 43	14 16	2 82	290	46 50
28	28	M	1 60	12 66	2 03		

TABLE II—*concl'd*

Number	Age in years	Diet	Surface area square metres	B Blood urea nitrogen mg per 100 c c	V Volume of urine c c per minute	U Urea nitrogen in urine mg per 100 c c	Maximum clearance, $\frac{UV}{B}$ c c
20	28	V	1 50	9 76	3 37	120	41 43
30	28	M	1 73	9 38	2 86	127	38 72
31	20	V	1 67	13 62	4 13	140	42 45
32	20	ML	1 62	13 20	2 33	227	40 01
33	20	V	1 70	11 11	2 42	185	40 30
34	30	M	1 35	10 10	2 75	108	29 41
35	30	V	1 83	12 10	2 43	170	35 68
36	34	M	1 82	11 87	2 56	187	40 00
37	37	M	1 76	9 94	2 07	249	52 10
AVERAGE	26		1 57	12 04	2 84	177	40 01

TABLE III

‘Standard clearance’ values in 73 normal young Indians

Number	Age in years	Diet	Surface area square metres	B Blood urea nitrogen mg per 100 c c	V Volume of urine c c per minute	U Urea nitrogen in urine mg per 100 c c	Standard clearance, $\frac{U\sqrt{V}}{B}$ c c
1	20	M	1 68	14 60	0 62	646	34 83
2	20	ML	1 72	12 82	0 75	609	41 14
3	20	V	1 81	14 02	0 66	526	30 47
4	20	V	1 73	10 95	0 52	441	29 04
5	20	M	1 62	12 50	1 07	364	30 11
6	21	ML	1 30	12 04	0 64	449	29 83
7	21	V	1 65	10 20	1 82	217	28 70

*Blood Urea Clearance in Normal Indians*TABLE III—*contd*

Number	Age in years	Diet	Surface area square metres	B Blood urea nitrogen mg per 100 c c	V Volume of urine c c per minute	U Urea nitrogen in urine mg per 100 c c	Standard clearance, $\frac{U \sqrt{V}}{B}$ c c
8	21	M	1 64	10 63	1 92	194	25 29
9	21	M	1 66	14 08	0 64	505	28 69
10	22	M	1 55	12 82	1 02	441	34 74
11	22	V	1 36	12 81	0 99	316	24 54
12	22	M	1 75	13 90	0 55	724	38 60
13	22	V	1 80	13 98	0 51	403	20 58
14	22	V	1 48	12 91	0 83	446	31 47
15	23	V	1 59	12 33	0 78	304	21 77
16	23	V	1 73	13 33	0 39	652	30 52
17	24	M	1 45	12 11	0 23	776	30 76
18	24	M	1 49	13 78	0 59	664	37 00
19	24	V	1 53	13 16	0 79	252	17 02
20	24	M	1 60	9 34	0 21	580	28 44
21	24	V	1 79	12 03	1 15	353	31 46
22	24	V	1 83	12 33	0 54	560	33 38
23	25	M	1 50	12 04	0 37	733	37 02
24	25	M	1 40	11 50	1 94	210	25 44
25	25	V	1 38	14 02	1 83	298	28 76
26	25	V	1 59	11 40	0 58	437	29 21
27	25	V	1 51	13 88	0 70	592	35 70
28	26	M	1 41	13 75	0 38	930	41 66
29	26	V	1 56	13 76	1 93	383	38 66
30	26	V	1 58	13 51	0 90	450	31 61
31	26	V	1 57	11 05	1 88	253	31 44
32	26	V	1 85	10 57	0 34	549	30 28
33	26	V	1 55	11 29	1 31	279	28 06

TABLE III—*contd*

Number	Age in years	Diet	Surface area square metres	B Blood urea nitrogen mg per 100 c c	V Volume of urine c c per minute	U Urea nitrogen in urine mg per 100 c c	Standard clearance, $\frac{U \cdot V}{B}$ c c
34	26	ML	1.66	12.03	1.39	212	20.76
35	27	ML	1.63	13.60	1.43	359	31.56
36	27	V	1.71	13.63	0.72	558	34.71
37	27	V	1.65	11.94	0.75	487	35.32
38	27	V	1.70	12.98	1.75	373	38.00
39	27	M	1.62	12.12	0.95	448	36.04
40	27	V	1.60	10.63	1.20	285	29.36
41	27	ML	1.45	10.42	0.61	319	23.23
42	27	V	1.61	12.50	0.77	394	27.64
43 ⁺	27	ML	1.73	9.70	0.83	367	34.49
44	28	ML	1.64	13.48	0.74	500	31.90
45	28	ML	1.63	13.88	0.58	609	33.43
46	28	ML	1.40	13.24	0.42	766	37.49
47	28	ML	1.37	10.93	0.36	595	32.66
48	28	ML	1.66	11.36	1.23	406	39.63
49	28	V	1.47	12.98	1.15	303	25.02
50	28	V	1.64	12.50	0.45	455	24.42
51	28	M	1.52	12.87	1.13	304	25.11
52	28	V	1.56	10.47	0.44	539	34.13
53	28	V	1.62	13.29	0.58	583	33.47
54	28	V	1.59	11.49	0.52	413	25.91
55	29	ML	1.68	13.61	0.47	667	33.62
56	29	V	1.85	11.58	0.98	429	36.68
57	29	V	1.57	10.15	1.05	389	39.28
58	29	M	1.60	10.38	0.63	519	39.70
59	29	ML	1.58	13.80	0.74	700	43.62

TABLE III—concl'd

Number	Age in years	Diet	Surface area square metres	B Blood urea nitrogen mg per 100 c c	V Volume of urine c c per minute	U Urea nitrogen in urine mg per 100 c c	Standard clearance, $\frac{U\sqrt{V}}{B}$ c c
60	20	V	1 50	12 55	1 14	460	39 15
61	29	M	1 62	13 80	0 34	826	34 90
62	29	M.	1 62	13 70	0 99	425	30 87
63	30	V	1 65	12 12	0 81	341	25 32
64	30	M	1 65	13 89	1 12	350	26 66
65	30	V	1 50	12 77	0 42	631	32 02
66	30	V	1 90	10 73	0 80	268	22 33
67	30	V	1 57	10 01	0 75	403	31 99
68	30	M.	1 71	12 04	0 92	165	37 04
69	32	V	1 56	9 26	1 04	218	24 01
70	33	M	1 63	12 95	1 90	409	43 52
71	33	V	1 96	11 62	0 90	311	25 40
72	33	M	1 53	10 31	1 37	278	31 55
73	40	V	1 90	10 63	1 76	251	31 33
AVERAGE	26		1 61	12 28	0 897	454	31 50

BLOOD UREA CLEARANCE VALUES.

The average maximum urea clearance obtained was 40 01 c c (Table II). The individual values ranged between 28 59 c c and 52 10 c c. The standard deviation was 5 355 and the range of significant variation, viz 34 65 to 45 37, covered 68 per cent of the subjects.

The average body surface area of these subjects was 1 57 square metres. Moller *et al* (*loc cit*) have observed that, as the clearance varies in direct proportion to the body surface, a correction for body surface area should be applied to the clearance value. They have adopted as a standard unit a body surface area of 1 73 square metres (this being the mean of surface areas for a large number of American men and women of 25 years of age), and 75 c c the average maximum clearance value for Americans given by them corresponds to the standard body surface area of 1 73 square metres. Unfortunately no reliable and sufficient data of

this kind were available for Indians. For purposes of comparison, however, the average value of 40.01 c.c. obtained for Indians becomes 44 c.c. when converted to correspond to a surface area of 1.73 square metres ($\frac{40.01 \times 1.73}{1.57} = 44$). This maximum clearance value, viz. 44 c.c., obtained for the normal Indian subjects is about 59 per cent of the average value of 75 c.c. obtained for American subjects.

The average standard clearance value obtained was 31.50 c.c. (Table III). The individual values ranged between 17.02 c.c. and 43.62 c.c. The standard deviation was 5.707. Seventy-one per cent of the subjects were within the range of the significant variation, viz. 24.79 to 37.29.

The average standard clearance value, viz. 31.5 c.c., for the 73 normal Indian subjects with an average surface area of 1.61 square metres would become, if converted to correspond to a surface area of 1.73 square metres, the standard adopted by Möller *et al* (*loc cit*), 33.8 c.c. ($\frac{31.5 \times 1.73}{1.61} = 33.8$). This value of 33.8 c.c. or 34 c.c. obtained for the series of normal Indian subjects of the present study is about 63 per cent of the standard blood urea clearance value of 54 c.c. obtained for the American normal subjects by Möller *et al*. Thus, it will be noticed that the blood urea clearance values C_m and C_s are about 60 per cent of the normal figures for American subjects. Therefore the American standard cannot be used for Indians and if used would give erroneous results, e.g. a standard clearance value of 32 c.c. representing 94 per cent ($\frac{32 \times 100}{33.8}$) efficiency when 33.8 c.c. the average standard clearance value obtained for Indians is used, would represent only 60 per cent efficiency, if the American standard, viz. 54 c.c., is used instead.

INDIVIDUAL VARIATION IN CLEARANCE VALUES

Some of the subjects in the present study were examined again on different occasions and their blood urea clearance values measured. The results are summarized in Table IV.

Table IV shows the individual variation in urea clearance value to be small as was also observed by Möller *et al*. In spite of this, 'an erroneous impression would be created by the clearance formulæ if they were assumed to express with mathematical exactness the complete effects of all factors influencing urea excretion' (Möller *et al*, *loc cit*). Still it seems to be the case, as pointed out by MacKay (1929), that blood urea content and urine volume are the two factors which are in continuous action and appear to be ordinarily of chief importance in regulating the urea output. The conditions under which the test as devised by Möller *et al* is carried out, take care of the two factors and minimize the effect of the other possible influencing factors to a great extent.

TABLE IV

Blood urea clearance values of 7 normal subjects on different days

Number	Clearance value c c	Mean and standard deviation	Number	Clearance value c c	Mean and standard deviation
1	28.4	M 30.7 S D ± 4.7	4	21.8	M 23.6 S D ± 0.52
	35.0			24.1	
	25.2			31.2*	
	35.4			25.0	
	28.8				
2	35.3	M 32.9 S D ± 3.3	5*	39.7	M 37.2 S D ± 3.3
	31.3			35.1	
	29.0			41.1	
	36.0			32.9	
3*	34.7	M 35.9 S D ± 4.7	6	25.1	M 26.4
	32.0			27.7	
	41.1		7	34.5	M 33.3
				31.9	

* This denotes maximum clearance values when V was greater than 2 c c

The results of this study show that diet has an influence on the blood urea clearance value. Most of the subjects studied were from the province of Bombay. It is recommended that similar studies be carried out in different parts of India.

SUMMARY

1 It is shown that while the average blood urea nitrogen content (viz 12 mg per 100 c c) is the same in both the Indian and American or European normal subjects the average urinary urea nitrogen excreted in twenty-four hours by normal Indians (viz 4.65 g) is less than half of that excreted by normal Americans or Europeans (viz 11 g) although the average volume of urine excreted by both is the same (1,300 c c).

2 The above findings showed that the use of American standards for evaluating the kidney function of Indians would give misleading results, since blood urea clearance is a relationship between urea in blood and that excreted in urine in a given space of time.

3 The blood urea clearance standards for Indians are worked out by examining urea clearance in 110 healthy young Indians from Bombay province with the following results —

Averages and range of variation in normal findings of 110 healthy young Indians

		Mean cc per minute	Maximum cc per minute	Minimum cc per minute.	Standard deviation	Surface area square metres	Clearance corresponding to surface area of 1.73 square metres
Maximum clearance	$\frac{UV}{B}$	40.01	52.10	28.59	± 5.35	1.57	44.0
Standard clearance	$\frac{U\sqrt{V}}{B}$	31.50	43.62	17.02	± 5.71	1.61	33.8

4 The average maximum clearance and the average standard clearance values obtained for the normal Indians were 44 cc and 33.8 cc respectively. These values are about 60 per cent of those given for normal Americans (maximum clearance 75 cc, standard clearance 54 cc).

5 Some normal individuals were examined on different occasions and the variation in the blood urea clearance values studied.

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DIFFERENCES IN THE RATE OF CHLORAL CLEARANCE IN BLOOD IN NORMAL AND LIVER-DAMAGED DOGS

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WHILE engaged in devising experiments for the determination of the detoxicating efficiency of the liver with special reference to its conjugation mechanism (Mukerji and Ghose, 1939a, b, 1940a, b, c), it was noticed that when a glucuronogenic drug like chloral hydrate was administered to a liver-damaged animal, the whole of the drug was not completely conjugated with glucuronic acid to form the non-toxic urochloralic acid, as is the case in normal healthy animals, but a small part of the drug was being excreted unchanged as *free chloral*. This observation led to the assumption that a damaged liver probably failed to elaborate glucuronic acid and carry out the protective conjugation process to the same extent as a normal healthy liver could do. Such a state would naturally be reflected in a significant increase, in both blood and urine, of free chloral in liver-damaged animals. In a previous communication (Mukerji and Ghose, 1940a), it was shown that chloral hydrate administration in doses of 200 mg/kg brought about a significant and well-marked increase in the level of free chloral excretion in the urine of dogs in whom experimental liver damage was induced by hepatotoxic doses of carbon tetrachloride. The present paper concerns itself with a study of the comparative rate of free chloral concentration in the blood of normal and liver-damaged dogs. The results obtained appear to lend strong support to our assumption that free chloral leakage in blood and urine following the administration of a known dose of chloral hydrate is roughly proportional to the degree of liver damage.

EXPERIMENTAL

Animals and their treatment—Young healthy dogs weighing on an average between 6.0 kilo and 7.0 kilo were chosen. Chloral hydrate, in doses of 70 mg/kg, 100 mg/kg, 150 mg/kg and 200 mg/kg, was administered through a stomach tube to each of these animals on different days. Following chloral administration, 1 c.c. samples of venous blood were drawn from one of the hind-limb veins at intervals of 30 minutes for a period of six to eight hours. The samples were then analysed for their free chloral content according to the method of Friedman and Calderone (1934). After a number of preliminary experiments (see Graph 1), it was found that the free chloral concentration in blood reached its maximum, about 45 minutes to 60 minutes, after the drug was administered. Incidentally, this was also found to be the period when the maximum degree of somnolence appeared to supervene in the animals as a result of the drug. In subsequent experiments, samples of blood were collected mostly during the period of maximum concentration of the drug in the blood (i.e. 45 minutes after drug administration). After a number of trials it was noticed that although the greater part of the drug was excreted from the blood within ten to twelve hours following an administration, small traces could yet be detected for a number of days, usually extending to a fortnight. In order to avoid any fallacy in subsequent experiments due to the amount thus present, a control sample of blood was taken before each fresh administration of the drug.

After the rate of the chloral clearance in blood had been ascertained in case of normal healthy dogs, liver damage was induced in these animals with toxic doses of carbon tetrachloride as reported before (Mukerji and Ghose, 1940a, b). At first a very mild grade of damage was brought about and the rate of chloral clearance was determined, as in the case of normal animals, after the administration of identical dosages of chloral hydrate.

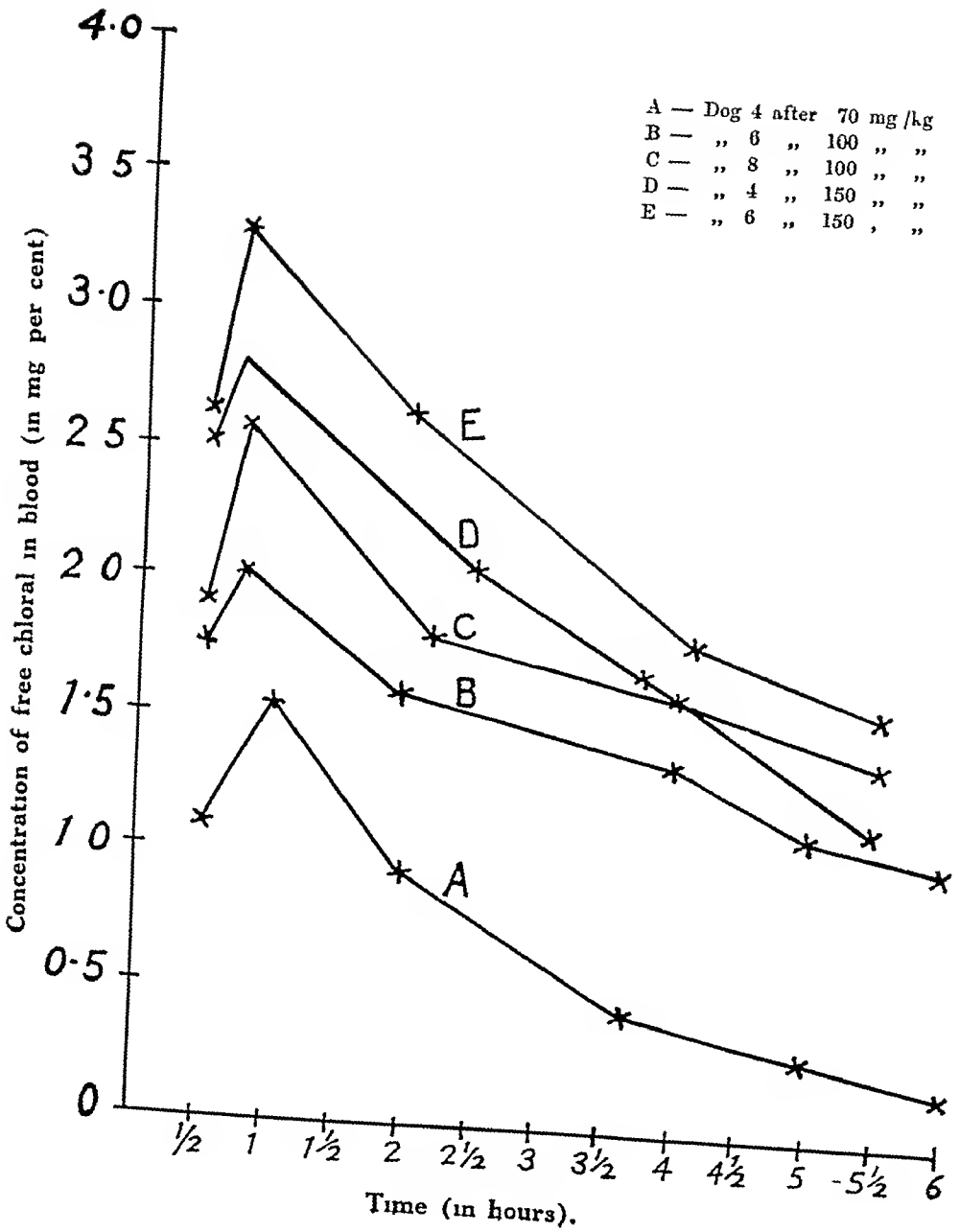
The animals were then subjected to repeated toxic doses of carbon tetrachloride so as to bring about an advanced state of liver injury. Rates of chloral clearance in blood were again ascertained under these conditions.

Carbon tetrachloride administration was then completely discontinued and the rates of recovery of the damaged organs were gauged from time to time by observing the chloral clearance in blood.

Test for free chloral—The method described by Friedman and Calderone was used. The colour reaction is more or less specific for compounds of the R—C—Halogen₃ group, and is consequently obtainable only with free chloral $[\text{CH}(\text{OH})_2\text{CCl}_3]$. After conjugation with glucuronic acid, chloral does not respond to this test. The details are briefly stated below—

‘One c.c. of blood is taken in a test-tube and is de-proteinized according to the method of Fohn and Wu by the addition of 8 c.c. of $\frac{\text{N}}{12} \text{H}_2\text{SO}_4$ and 1 c.c. of 10 per cent sodium tungstate. The contents of the test-tubes are then thoroughly mixed by vigorous shaking, allowed to stand for 15 minutes and then filtered off. Five c.c. of the blood filtrate are then taken into a test-tube to which are added 2 c.c.

GRAPH I



of 40 per cent NaOH and 1 c.c. of colourless pyridine. After thoroughly mixing by brisk shaking, the test-tube is placed in a boiling water-bath where it is kept for exactly one minute and then cooled in an ice-bath for one minute. Five c.c. of distilled water are then added and the intensity of colour developed is determined by means of a photometer (Pulfrich type). The determination has to be completed within a couple of minutes after the dilution with distilled water, as the colour fades away very quickly. The results are then compared with a calibration curve made with chloral hydrate solutions of different strength from which the absolute figures are readily obtained.

RESULTS

In Table I are shown the maximum concentrations of free chloral in blood, attained after the oral administration of chloral hydrate in doses of 70 mg/kg, 100 mg/kg, 150 mg/kg, and 200 mg/kg. It will be noticed that with an identical dose of the drug, the blood concentration shows a very uniform range in all the experimental animals. With gradually increasing doses of the drug, the free chloral concentration in blood also tends to increase but the slope of the curves (Graph 1) is more or less similar.

TABLE I

Showing the blood concentration of free chloral in normal healthy animals, following the oral administration of chloral hydrate in different doses

Dog number	Maximum concentration of free chloral in blood (mg. per cent)			
	Dose of chloral hydrate administered orally			
	70 mg/kg	100 mg/kg	150 mg/kg	200 mg/kg
1 (6.3 kg)	1.15	2.38	2.80	3.82
4 (6.3 kg)	1.54	2.25	2.90	3.56
6 (6.4 kg)	1.15	2.00	3.32	4.15
8 (6.0 kg)	1.46	2.54	3.00	4.20

The effect of liver damage on the blood chloral concentration is shown in Tables II and III. Table II shows the effect of comparatively minor degrees of liver damage, brought about by the administrations of only four to five doses of carbon tetrachloride. Here, although the increase of blood chloral concentration is not very marked yet it is still significant. The effect of severe liver damage on the chloral concentration in blood is evident from the figures shown in Table III. There is a well-marked rise of the blood chloral concentration indicating a tendency to failure of glucuronide conjugation mechanism.

TABLE II

Showing the effect of early liver damage (caused by four to five administrations of carbon tetrachloride, each administration 2 c.c./kg) on the blood concentration of free chloral, following the oral administration of chloral hydrate in different doses

Dog number	Maximum concentration of free chloral in blood (mg per cent) Dose of chloral hydrate administered orally			
	70 mg/kg	100 mg/kg	150 mg/kg	200 mg/kg
1	2.00	3.00	4.00	4.90
4	2.00	3.32	4.00	5.00
6	1.83	3.00	5.00	6.50
8	2.25	3.12	4.20	5.32

TABLE III

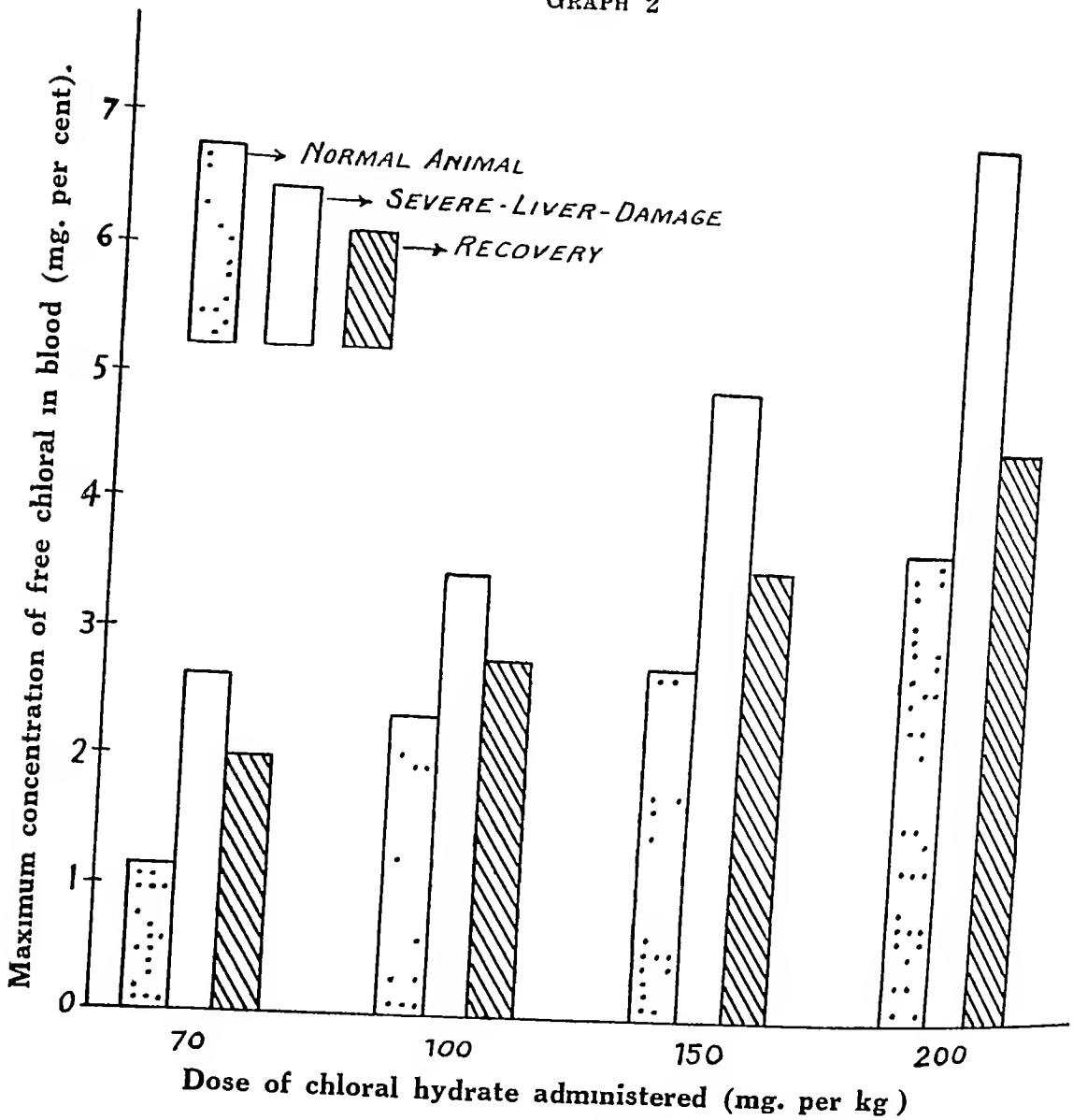
Showing the effect of severe liver damage (caused by the oral administration of 20 to 30 doses of carbon tetrachloride, each dose between 1 c.c./kg and 2 c.c./kg) on the blood concentration of free chloral following the oral administrations of chloral hydrate in different doses

Dog number	Maximum concentration of free chloral in blood (mg per cent) Dose of chloral hydrate administered orally			
	70 mg/kg	100 mg/kg	150 mg/kg	200 mg/kg
1	2.65	3.50	5.00	7.00
4	2.90	3.65	5.50	7.50
6	2.69	4.08	7.33	9.00
8	2.85	4.00	6.30	8.50

The effect of recovery of the damaged liver on the blood concentration of free chloral is seen well from Graph 2 showing the condition one and a half months after stoppage of chloral administration in dog 1. Carbon tetrachloride administrations were completely discontinued and observations on blood chloral levels were made from time to time. In case of all the animals a significant diminution gradually tending towards the normal could be clearly seen. There

was also a marked improvement in the general condition of the animals as evidenced by weight increase, improvement of appetite and improvement in skin condition due to diminished excretion of chloral via skin

GRAPH 2



DISCUSSION.

From the foregoing data it is evident that, in liver-damaged dogs, the free chloral concentration in blood following the oral administrations of chloral hydrate in different doses, is definitely greater than in apparently normal and healthy animals

This increase is demonstrable not only in the dogs which have been exposed to carbon tetrachloride for a very long time and whose livers have been very severely damaged, but also in those whose livers have undergone a comparatively minor damage by the influence of the poison for a short period. Even after four or five administrations of carbon tetrachloride, a comparatively higher rise than the average normal blood chloral concentration becomes evident. This leakage of free chloral appears with such regularity under experimental conditions of liver injury that it seems logical to claim that this criterion can be used as a measure of the hepatic efficiency in clinical practice. The method of colorimetric determination of free chloral in blood is fairly simple and chloral hydrate, being a hypnotic drug, can be easily administered to many patients in the usual course. In view of the fact that free chloral leakage would measure the de-toxicating ability of the liver cells, this test deserves careful scrutiny and consideration in the hands of the clinicians.

SUMMARY

1 Chloral hydrate in doses of 70 mg/kg, 100 mg/kg, 150 mg/kg and 200 mg/kg, was administered to apparently normal healthy dogs and the concentrations of free chloral in the blood determined by a comparatively simple but sensitive colour test.

2 Hepatotoxic doses of carbon tetrachloride were then orally administered to these animals in order to bring about varying degrees of liver damage, and the effects of identical doses of chloral hydrate on the blood chloral concentration were studied. In both recent and long-standing liver injury a significant increase in the concentration of free chloral in the blood could be demonstrated.

3 It is suggested that the difference in the level of free chloral concentration in blood, following the oral administration of a definite dose of the drug, may be used as a measure of the de-toxicating efficiency of liver.

ACKNOWLEDGMENT

The authors wish to express their grateful acknowledgment to Brevet-Colonel Sir Ram Nath Chopra, C I E, I M S (*Retd*), Director of the Laboratory, for his guidance and encouragement throughout the course of this research.

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EXPERIMENTAL LIVER AND BILIARY DAMAGE AND SERUM PHOSPHATASE

BY

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ROBISON (1923) first demonstrated the presence of an enzyme, which he termed 'phosphatase', in bone and ossifying cartilage, and gave experimental evidence to show that this enzyme is intimately related to the process of bone formation. Since then it has been studied in the blood and its quantitative estimation in blood serum and plasma has been of considerable diagnostic value in a limited group of pathologic conditions affecting bony tissues, such as local bony atrophy, generalized osteoporosis, osteomalacia, rickets, Paget's disease, etc (Kay, 1930, 1932). Roberts (1930) undertook a systematic study of the blood phosphatase content in disease states and observed high values in the three cases of jaundice which he had included in his series. He noted, moreover, a striking difference in the phosphatase content of the plasma of the two cases of obstructive and the one case of non-obstructive jaundice studied. This observation led him, in a subsequent work (Roberts, 1933), to study the phosphatase values in a series of 52 cases of jaundice, 21 of which were obstructive and 31 non-obstructive. He concluded that by means of the phosphatase level of the blood, 'toxic, infectious and catarrhal jaundice may be readily distinguished from jaundice of the obstructive type'. Subsequent workers, Bodansky and Jaffe (1934), Armstrong, King and Harris (1934) and Greene, Shattuck and Kaplowitz (1934), also observed a rise of phosphatase value in all cases of jaundice other than that of hemolytic origin. Bodansky and Jaffe (*loc cit*) ligated the common bile duct of a dog and noted the progressive elevation in the serum phosphatase paralleling the rise in the serum bilirubin. They believed, however, that the determination of plasma phosphatase was of no value in differentiating between obstructive and non-obstructive jaundice.

Freeman, Ping Chen and Ivy (1938), while studying in experimental animals the elevation of serum phosphatase in jaundice, found that practically in all types of artificially induced liver injuries, e.g. by leptospiral infection, by carbon tetrachloride poisoning, by partial or complete obstruction of the common bile duct or by partial hepatectomy, there was increase in the values of serum phosphatase. They were of the opinion therefore that the rise in phosphatase values could be attributed more to after effects of liver injury than to any primary bony involvement. Gutman *et al* (1940), on the other hand, after studying about 300 cases of different types of jaundice, maintained that liver injury might not be the more important factor in this process as (1) there is no rise in phosphatase in certain cases of jaundice (hæmolytic type) and (2) there is definite rise of phosphatase value in cases of rickets, congenital atresia of the common bile duct and in certain bone diseases which have no relationship whatsoever with liver injury.

In view of the interest attaching to the subject, not only with respect to the differential diagnosis of different types of jaundice, but also as regards the mechanism of production of a high serum phosphatase level and the role of the liver in this process, it was thought desirable to study the problem further.

EXPERIMENTAL METHODS

The main series of experiments were carried out on dogs. Young healthy pups were kept in metabolism cages under a more or less constant daily diet. After the usual period was allowed for acclimatization to the conditions of the laboratory, blood samples were obtained from one of the leg veins for the determination of normal phosphatase values in duplicate. The animals were then divided into two groups, keeping two animals out of the group to serve as controls. To the first group of dogs, carbon tetrachloride in doses of 0.5 c.c. per kg. body-weight was administered to bring about a condition of hepatocellular damage of the type reported by Mukerji and Ghose (1940). In the second group, experimental biliary obstruction was produced by tying the bile duct through a laparotomy wound. Under phenobarbital-ether anaesthesia, laparotomy was performed with all aseptic precautions and the common bile duct and cystic duct were tied by sterile silk ligatures in two or three places. The abdominal wound was closed and the dogs allowed to recover. The operation involved considerable technical difficulties and several animals were lost due to sepsis and other causes during the post-operative period. In a few animals however satisfactory 'survival experiments' were possible.

Serum phosphatase determinations were carried out by the modified method of Bodansky (1937).

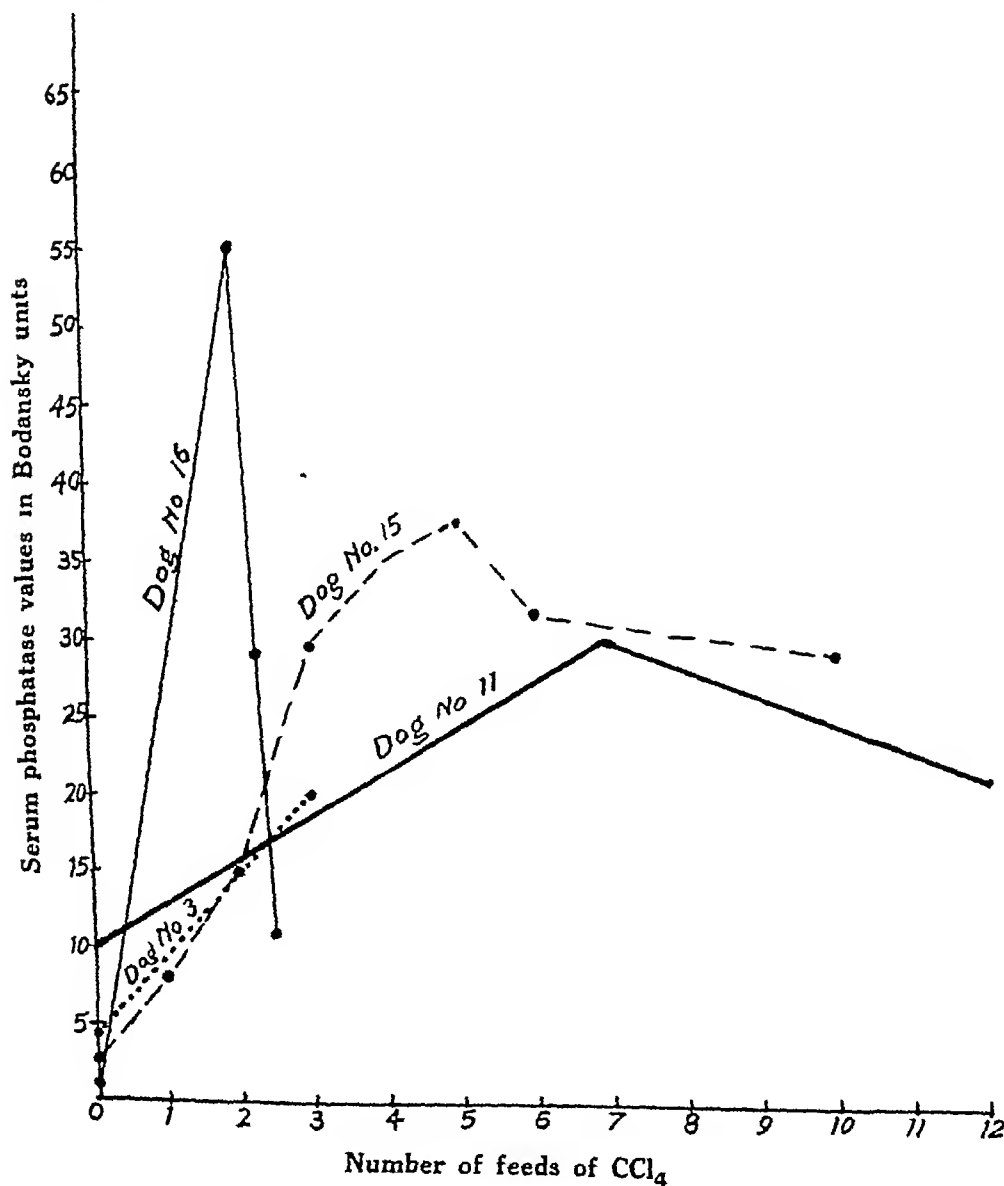
RESULTS

The normal serum phosphatase values were determined in 13 dogs of the series during the first week before the starting of the experiments. The figures ranged between 2.1 and 5.4 Bodansky units per 100 c.c. blood serum with an average of 3.68 ± 1.45 units.

Phosphatase values following the administration of carbon tetrachloride were then determined in the first group of animals of the series. The typical results obtained are shown in Graphs 1 and 2. Gardener *et al* (1925) had shown that as

GRAPH 1

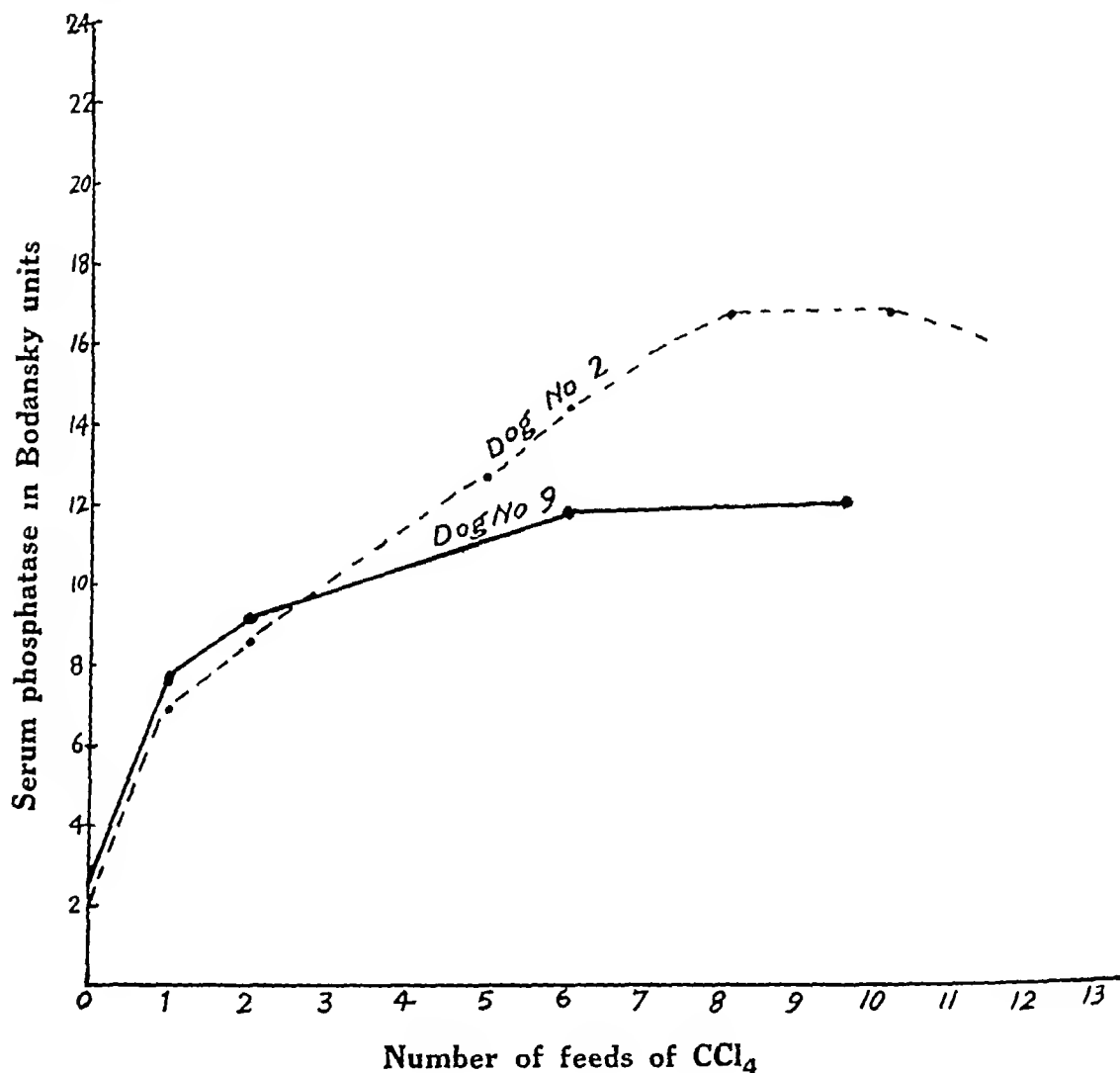
Hepatotoxic drug CCl_4 (acute liver damage by single administration)



early as 24 hours after the administration of carbon tetrachloride in dogs there was demonstrable injury to the liver cells. This is reflected in the phosphatase values which have, in general, shown an increase following even the first administration of carbon tetrachloride. After two or three administrations of this liver poison, there

GRAPH 2

Hepatotoxic drug CCl_4 (chronic liver damage by prolonged feeding)



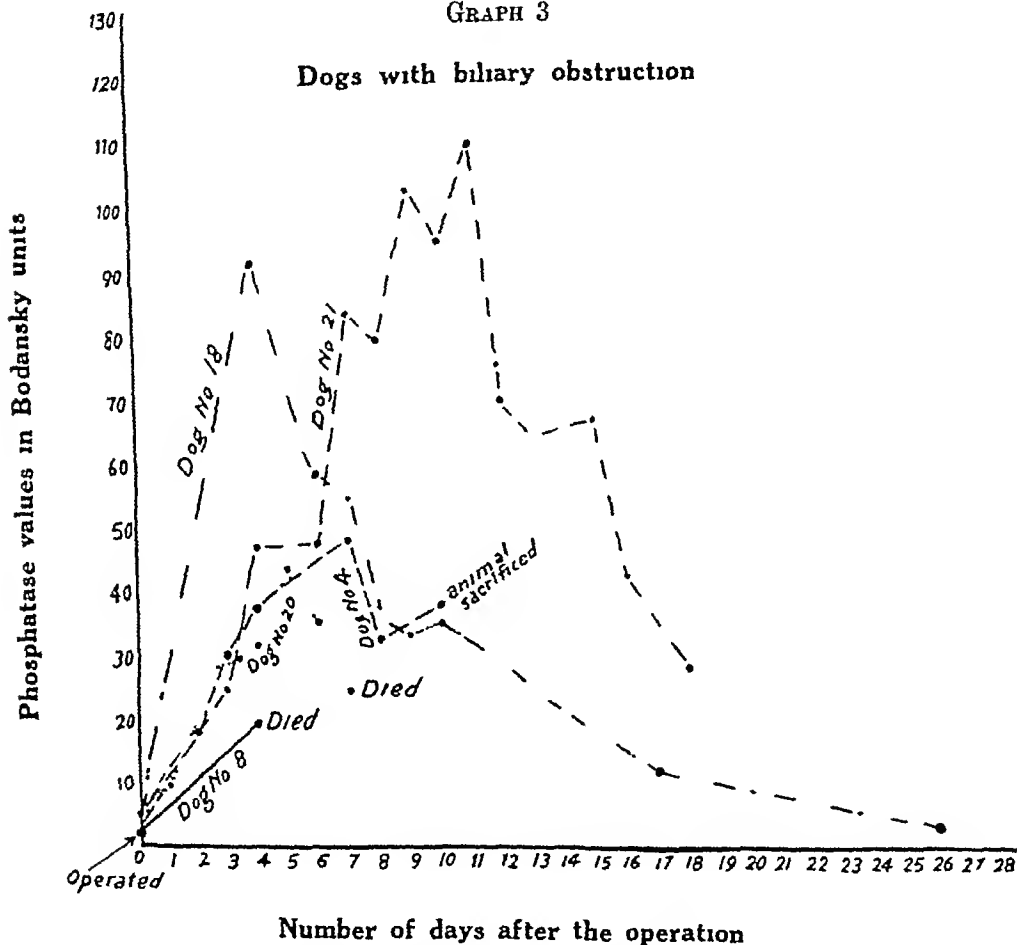
is a definite and consistent increase in the serum phosphatase in all dogs, though the degree of response has varied in individual animals. That serum phosphatase has a definite relationship to liver damage is very well seen in the case of dog No 16 where an acute damage was purposely brought about by a heavy dose of carbon tetrachloride. The serum phosphatase level has gone up to a very high value in

this animal in marked contrast to the picture presented by other experimental animals

In Graph 2 are represented the phosphatase values of two dogs where chronic liver damage was brought about by repeated and prolonged administration of carbon tetrachloride in small doses extending over 2 to 3 weeks. The phosphatase values in these dogs never showed a marked elevation to the same extent as seen

GRAPH 3

Dogs with biliary obstruction



in those animals in which recent and acute poisoning was induced. The highest phosphatase figure recorded in this series was 17.8 units as against 55 units in cases of acute poisoning. After an initial sharp response to any liver injury, the serum phosphatase tends to come to a more or less steady level.

The data in connection with the second series of experiments by artificially obstructing the biliary passages are presented in Graph 3. It may be pointed out

here that the operative obstruction brought about in our series of dogs may be considered *as only partial*. Unlike many other similar experiments reported in the literature, we have not succeeded in producing complete biliary obstruction in these animals with demonstrable jaundice and cholemia. Dogs apparently have a large number of small accessory bile channels and even when the common bile duct and the cystic duct are tied in more than two to three places with silk ligatures, bile seems to escape into the intestines after 6 to 7 days, as evident from the colouring of the stools and urine. Presumably some accessory bile duct remained untied under the conditions of our operative experiments.

From a reference to Graph 3, it will be seen that the phosphatase response in biliary obstruction is most intense and marked, a value of as much as 90 to 110 Bodansky units being recorded in dog Nos. 18 and 21 in which observations could be continued for a fairly long period. As the obstruction to biliary flow tended to decrease due possibly to the dilatation of accessory channels, the phosphatase values tended to diminish. In dog No. 18 which was followed up for a prolonged period, the normal range was reached after the third week.

DISCUSSION

These data are essentially in agreement with those reported by Bodansky and Jaffe (*loc cit*), Herbert (1935) and Greene, Shattuck and Kaplowitz (*loc cit*). As in the case of the data presented by Greene *et al* (*loc cit*) and Cantarow and Nelson (1937), the phosphatase values obtained in our series exhibit a wide overlapping in obstructive and hepatocellular jaundice, though in the former type there is generally a tendency to higher phosphatase values being reached. This observation is contradictory to the findings of Roberts (1933) and Rothman, Meranze and Meranze (1936) who believed that there is a rather sharp line of demarcation in this regard between the two types of jaundice. Rothman and his associates found values greater than 10 units per 100 c.c. for obstructive jaundice and 10 units or less with hepatocellular jaundice. Although our data definitely indicate that values above 25 Bodansky units can only be associated with some form of biliary obstruction, either partial or complete, it appears that values ranging between 10 and 25 units can occur in different phases of both hepatocellular damage and obstruction in biliary flow. Values below 10 units are again seldom met with in obstructive involvement of the biliary tract. A sharp delimitation between obstructive and non-obstructive jaundice on the basis of phosphatase determination therefore is not possible for at least a small group of cases in which the phosphatase values lie between 10 and 25 units and as such, phosphatase determinations would have comparatively limited practical significance from the standpoint of differential diagnosis. There is no doubt however that it affords a very good indication of the state of the liver and the biliary system in general, and probably in a fairly large proportion of cases would serve as a reliable guide in gauging the type and extent of liver injury and biliary stasis.

It seems futile in the present state of knowledge to theorize regarding the possible mechanism of production of the increase in serum phosphatase in liver

injury, in biliary obstruction and in bony involvements. The subject has been recently reviewed by Herbert (*loc cit*) and also by Cantarow (1936). It appears to be clear, however, that this increase cannot be explained on the basis of a simple obstruction to the flow of bile, either extrahepatic or intrahepatic and its reabsorption. This hypothesis is contradicted by observations of Greene and his colleagues (*loc cit*) in portal cirrhosis and also by Cantarow and Nelson (*loc cit*) who showed that marked elevation of serum phosphatase level could be accompanied with little or no increase in serum bilirubin. In congenital obliteration of bile ducts, normal phosphatase activity has also been shown to exist which also goes against the viewpoint of the association of phosphatase with bile. We are therefore of opinion, in agreement with Gutman *et al* (*loc cit*), that liver injury probably plays a very important rôle in this process.

This hypothesis is supported by our own observations, as well as the findings of a large number of workers in the field, that experimental liver damage brought about by any means is always associated with a high serum phosphatase level. In various types of clinical conditions characterized by an acute or chronic inflammatory reaction in the liver, serum phosphatase has further been found to be constantly elevated, the degree depending on the intensity of the pathological lesion induced. This hypothesis can further explain the association of an elevated serum phosphatase level in certain types of bony diseases, e.g. rickets. In this condition a certain amount of enlargement of the liver has almost always been clinically observed and it is not improbable that liver is the organ primarily at fault in rickets. It is conceivable that liver injury due to some unknown chemical or bacteriological toxin in rickets would give rise to an increase in serum phosphatase, which in turn would raise the total inorganic phosphorus in the blood and lower the calcium balance of the system. The bone, being the most important focus of absorption of Ca and P, is thus affected leading to varying degrees of local de-calcification and skeletal troubles. In order to support this view experiments *in vitro* were tried to see if a possible relation between phosphatase and vitamin D could be established. It was found that vitamin D (cod-liver oil) had an inactivating effect on the enzyme. It was further corroborated by Correl and Wise (1939), who have conclusively proved, by their experiments on chicks, that the anti-rachitic vitamin had a marked inhibitory effect on the activity of phosphatase. It was noticed that the fall in the activity of phosphatase was almost directly proportional to the healing of rickets in chicks, thus showing that with the inactivation of phosphatase, the Ca-P balance in the blood returned to normal and healing of rickets supervened.

The part played by liver in the Ca-P metabolism does not appear to have attracted sufficient attention. The fact that high phosphatase values are attained in all skeletal diseases and that a similar rise in phosphatase value is also observed in liver damage caused artificially or otherwise, leads us to speculate on the possibility of the liver being the seat of primary disturbance, the manifestations of which are observed in the bones. Work is in progress where an attempt is being made to show that liver damage can lead to the formation of rickets or skeletal disturbances. This is expected to throw more light on the rather obscure relationship between bony involvement and liver disease.

SUMMARY AND CONCLUSION

1 The blood serum phosphatase values have been studied, by the modified Bodansky method, in a series of dogs in which experimental liver damage and biliary obstruction were induced, the former by administration of hepatotoxic drugs such as CCl_4 and the latter by putting ligatures in the common bile duct

2 The phosphatase values in cases of biliary obstruction were generally found to be greater than 25 Bodansky units, while in primary liver injury the values seldom exceeded 20 Bodansky units. Phosphatase values of 30 Bodansky units or above may therefore be always considered as definitely diagnostic of biliary obstruction. Values between 10 and 20 Bodansky units may be obtained in both obstructive and non-obstructive (hepatocellular damage) types

3 This test therefore, while indicative of the general status of the liver and biliary system, cannot be considered as a *sure test* for the differential diagnosis of obstructive and non-obstructive types of jaundice

4 The possible causes underlying the phenomenon of the elevation of serum phosphatase have been discussed. The idea that high serum phosphatase level in biliary obstruction is probably due to re-absorption of bile itself is discounted and support has been given to the viewpoint that liver damage is probably the primary factor involved. On the basis of this assumption, an explanation has been offered with regard to the association of liver injury, phosphatase rise and skeletal involvement

ACKNOWLEDGMENTS

It is a pleasure to place on record our indebtedness to Brevet-Colonel Sir Ram Nath Chopra, C I E , I M S (*Retd*), for advice and guidance. To Dr S K Chatterjee, B Sc , M B (Cal), F R C S (Edin), D L O (Lond), Visiting Surgeon, Campbell Medical School and Hospitals, who performed most of the bile duct ligature operations for us, our thanks are also hereby tendered

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COMPETITION OF PROTEIN SUBSTRATES TOWARDS PROTEOLYTIC ENZYMES

BY

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IN connection with the problem of the mechanism of destruction of insulin in blood, a detailed study of the proteolytic system in the various fractions of blood (red corpuscles, plasma, leucocytes and platelets) was undertaken by us (Iyengar and Scott, 1940) at the Connaught Laboratories, Toronto. The platelets were found to possess the highest tryptic activity in comparison with the other fractions of blood, calculated on the total solids basis. A suspension of platelets after separation of red blood cells, leucocytes and plasma was precipitated by four volumes of acetone and dried. This preparation, when suspended in M/15 phosphate buffer of pH 8.4 and incubated at 37°C for 24 hours, showed considerable auto-digestion. The presence of trypsin in the platelets was thus demonstrated. The enzyme preparation (1) obtained by acetone precipitation contained the platelet proteins, which acted as a substrate for the proteolysis by the trypsin present. The trypsin from the platelets was also prepared by another method without acetone precipitation. The sediment obtained after the first centrifugation of plasma was suspended in citrated saline and kept in the refrigerator for a few days, the supernatant siphoned off, and the sediment again subjected to the same process twice or thrice. Finally, the sediment was frozen and dried in a sulphuric acid vacuum desiccator at room temperature. The dried platelets were extracted with M/15 phosphate buffer of pH 8.4 and centrifuged. The supernatant solution (2) served as the second enzyme preparation.

The action of the two enzyme preparations (1) and (2) from the platelets on pure insulin was studied primarily with a view to throw some light on the physiological destruction of insulin in the body. The results concerning this aspect of the problem has been communicated to the *Transactions of the Royal Society of Canada* (to appear shortly). During the course of this work, certain interesting observations throwing light on the question of the competition of the different protein substrates

when they are present in a mixture, to the same proteolytic enzyme, were made. The proteolytic action of the platelets enzyme (1) on the following proteins was studied by estimating the increase in the non-protein nitrogen in each digest. The results are briefly tabulated below —

	Increase in N P N in the whole digest, mg	Increase in diges- tion due to the added protein, mg
(1) Platelet powder 1 g suspended in 20 c c M/15 phosphate buffer of pH 8.4	9.48	
(2) 1 g platelet powder suspended in 20 c c of 1 per cent casein solution in the above buffer	18.88	9.40
(3) 1 g platelet powder suspended in 20 c c of 1 per cent insulin solution in the above buffer	9.04	Nil
(4) 1 g platelet powder suspended in 20 c c of 1 per cent suspension of plasma proteins in the same buffer	15.08	5.60

In all the above experiments, with the exception of (1), the reaction mixture consisted of two substrates, the platelet protein being common in all of them. The fact that the highest amount of digestion has taken place in (2) shows that casein is most susceptible to the enzyme attack, in comparison with the other protein substrates, plasma proteins and insulin. Next in order comes the plasma proteins. Insulin does not appear to have been attacked by the enzyme, as there was neither an increase in nitrogen, nor a decrease in physiological activity.

Insulin being a protein in nature is destroyed by all proteolytic enzymes, trypsin-kinase, pepsin and papain (Freudenberg *et al.*, 1930, 1932). The platelet enzyme has been shown by us to belong to the group of tryptases and hence should be naturally expected to destroy insulin. This apparently anomalous behaviour of the enzyme towards insulin can be explained on the basis of varying susceptibility to proteolysis of different proteins when present in mixtures. The reaction mixture in this case contained two substrates, the platelet proteins and insulin. It is likely that the enzyme being precipitated along with the platelet proteins during the preparation, has a great preference to split the associated proteins and in this competition of the two substrates, the insulin protein does not offer itself for attack.

When a proteolytic enzyme is added to a mixture of proteins, it is not possible to decide which of the two proteins has been attacked, since the criteria for judging the digestion are similar in the case of both the proteins, whichever method of estimation of proteolysis is followed. An approximate idea of the extent of hydrolysis of the individual proteins in a mixture can however be obtained in a few

cases by estimating the release of any one of the amino acids present in one of the substrates and not present in the other. Studies of this kind do not appear to have been made and therefore experimental evidence, for the competition of protein substrates when present in mixtures, to enzyme attacks, is lacking. The present study of insulin throws very interesting light on this problem. If the protein structure of insulin is not affected, the activity of insulin remains unchanged (Jensen, 1938). Hence, if any proteolysis has taken place, it should be followed by a decrease in physiological activity. This provides an alternative method of finding out whether proteolysis of insulin has taken place when present in a mixture with another protein substrate. If, in such a mixture of substrates, non-protein nitrogen is released as a result of enzyme action, while at the same time physiological activity remains intact it can safely be inferred that the enzyme has attacked the other protein exclusively.

When the enzyme preparation (2) was incubated with insulin, a definite proteolysis of the insulin protein and also the destruction of physiological activity, was observed. Two explanations can be offered for this apparently anomalous behaviour of the different enzyme preparations from the same source. In the acetone precipitated preparation, since the enzyme was simultaneously precipitated with the platelet proteins, it is likely that the trypsin is adsorbed on the protein. It is known that the adsorption of the enzyme on a substrate is the first stage of enzymic digestion. Hence, to such a preparation when insulin is added, the enzyme being already engaged by the other protein, is not in a position to digest the other added protein. A more plausible reasoning would be the different degrees of susceptibilities of different protein substrates when present in a mixture. The experiments with casein and plasma proteins lend experimental support to the latter hypothesis. The enzyme preparation (2) being a buffer extract of the dried platelets, contained little platelet protein in the solution. The reaction mixture being predominantly insulin and the trypsin from the platelets, a competition between the substrates did not exist and hence insulin protein lent itself to attack by the enzyme.

The possibility that the S-S groupings in the insulin molecule might be acting as an inhibitor to the enzymatic digestion of the other protein associated with insulin in the mixture, has also to be considered. An examination of the table shows that the amounts of non-protein nitrogen released in 1 and 3 are the same, indicating that the break-down of the platelet protein has not been in any way affected by the addition of insulin. The increase in non-protein nitrogen in 3 is essentially due to the break-down of the platelet protein since the physiological activity of mixture 3 has not been altered at all after incubation with platelet enzyme. It is therefore concluded that the results obtained throw direct light on the comparative susceptibilities of different proteins to enzymic break-down, when the former are present in mixtures.

It appears from the above experiments that among the proteins tested, insulin is the most resistant to tryptic attack when other proteins are present in reaction mixture. Proteins, which are easily digested by trypsin, appear to offer protection to insulin from enzymic proteolysis. A study of the digestion of insulin by pure

crystalline trypsin in the presence of high concentrations of other proteins easily susceptible to enzymic attack, will throw direct light on the above hypothesis. It is proposed to extend this work on the lines indicated.

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ALTERATIONS IN THE ELECTROCARDIOGRAPHIC FEATURES BROUGHT ABOUT BY DIGITALIS

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SOME controversy has taken place as to the changes brought about by digitalis on electrocardiographic features, especially with regard to the *T* wave. According to Pardee (1933) digitalis causes a diminution of the height of the *T* wave and a depression of the *S-T* interval. In some cases, only the *T* wave may be affected without materially altering the *S-T* interval. The *T* wave may thus become inverted without any appreciable change in the level of the *S-T* interval. The *Q R S* group is not materially altered with digitalis. A similar view with regard to the *T* wave was taken by Robinson and Wilson (1918) who regarded the inversion of the *T* wave as the first evidence of digitalis intoxication. On the other hand, Yacoel and Papanayotou (1927) and others found that the *T* wave remained upright and became taller after digitalis. According to Maher (1937), the effect of digitalis on electrocardiogram from moderate oral administration over a period of time is practically limited to a change in *S-T* segment and a mild increase in *P-R* conduction time. The *S-T* segment is depressed and becomes concave. The concavity gradually deepens until the *T* wave is obliterated. Maher himself never obtained a straight inversion of *T* wave by digitalis alone. Korth and Spang (1937) gave intravenous injections of toxic doses of digitoxin in dogs and studied the electrocardiographic changes resulting for several days up to three weeks. They observed two typical pathological changes in the electrocardiogram, depression of the *S-T* interval and the infarct type of *S-T*. Subsequently, the animals were killed and histological sections of the heart were made and examined. It was found that areas of necrosis were found in the heart muscle at various places. It was also observed that the extent of the necrosis was in proportion to the electrocardiographic variations in the *S-T* interval. The authors, however, concluded that the mere depression of the *S-T* interval did not correlate with a discernible injury in the

heart muscle Brams (1929) did not obtain inversion of the *T* wave in a series of experiments on dogs in which digitalis was administered in ascending doses until the death of the animal occurred

Brams and Gaberman (1931) experimented on nine adult volunteer patients who were convalescing from peptic ulcer, arthritis and the like conditions but who gave no history or evidence of cardiac disease Digifoline was given intravenously once a day in increasing doses until symptoms like nausea, vomiting and precordial distress were produced None of these subjects showed inversion of the *T* wave though other cardiac effects like slowing of the pulse rate, even partial heart block and transient auricular fibrillation, were produced

In the present investigation the effect of digitalis on the electrocardiogram was studied on the hearts of various animals

THE EFFECT ON PERFUSED FROG HEART

The frog (*Rana tigrina*) was killed by pithing and the heart was perfused with Bayliss' solution through the inferior vena cava After the heart continued to beat regularly for some time an electrocardiogram was taken with leads directly applied to the left auricle and the apex of the heart Tincture digitalis diluted in Bayliss' solution in proportion varying from 1 10,000 to 1 100 was then perfused There was no perceptible effect with a dilution of 1 10,000 But the dilution of 1 1,000 caused marked effect in the electrocardiogram, the effect increasing with the increase of the strength Plate XII, fig 1, indicates the action of digitalis on the perfused frog heart Fig 1*a* is the electrocardiogram taken while the heart was perfused with Bayliss' solution before digitalis perfusion was started The subsequent records *b*, *c* and *d* were taken at 5-minute intervals after starting the perfusion with 1 100 tincture digitalis in Bayliss' solution Certain features are to be noted The *P* wave which is not distinct even in the first record gradually disappears The *P-R* interval which is always prolonged in frog's electrocardiogram has not materially increased within the short period in which the *P* wave is discernible The *R-S* waves have not altered with the exception of a little diminution in the excursion in *b* Marked alterations are seen in the *S-T* interval and in the shape of the *T* wave The *S-T* interval has progressively become diminished in length and has become concave upwards, while the *T* wave has become markedly upright with a pointed apex in *b* and *c* It is seen that, as the perfusion with digitalis continues, the heart has gradually slowed Similar features were observed in the electrocardiogram of seven other frogs experimented upon

DIGITALIS AND MAMMALIAN ELECTROCARDIOGRAM

The action of digitalis on mammalian electrocardiogram was investigated in acute experiments on rabbits, dogs and cats A 1 20 dilution of tincture digitalis in Ringer's solution was slowly infused through the femoral vein of the anaesthetized animal Electrocardiograms were taken at short intervals till the animal died

PLATE XII

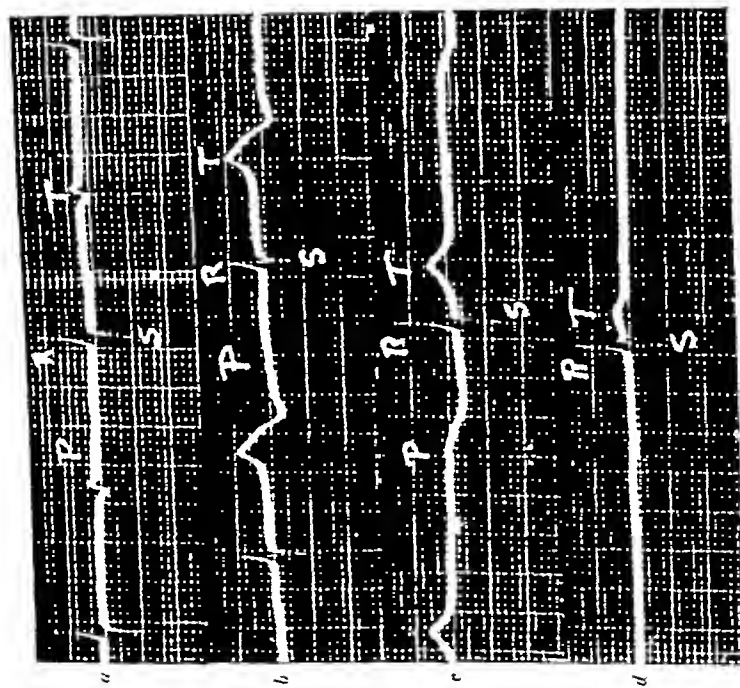


FIG 1—Electrocardiographic records of the hearts of an isolated, perfused frog heart, the leads being applied directly to the left auricle and the apex of the heart. *a* is record taken while the heart was perfused with Baylis' solution. *b*, *c* and *d* are records taken at 5 minute intervals after starting perfusion with 1:100 tincture digitalis in Baylis' solution.

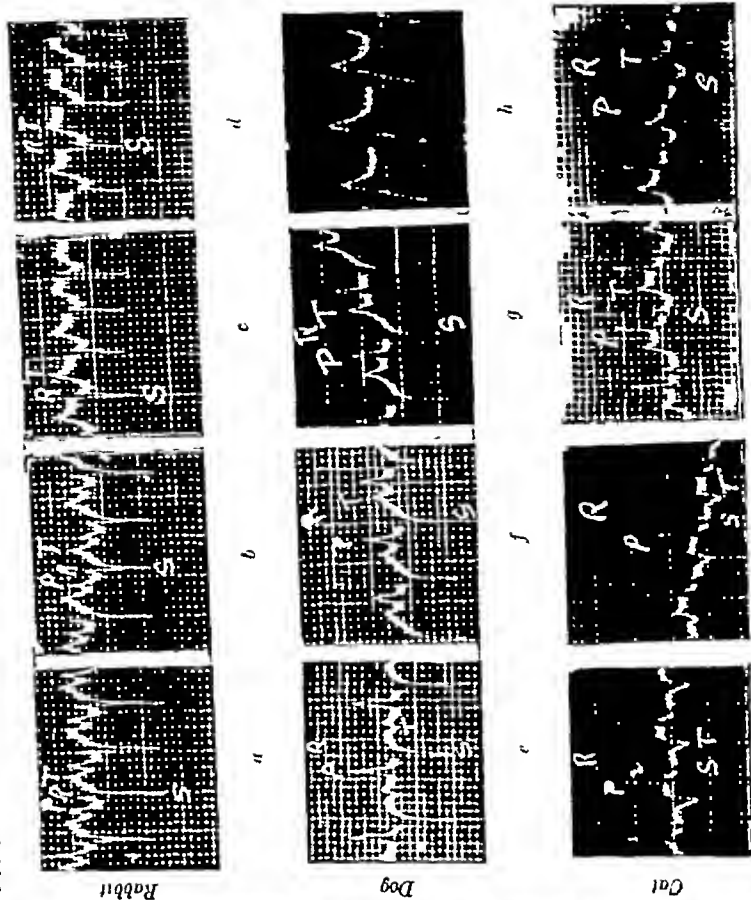


FIG 2—Electrocardiographic records obtained in rabbit, dog and cat, only lead 2 (right fore limb \rightarrow left hind limb) being shown. The initial record in each case is that taken while the animal was under the anesthetic and before the digitalis infusion was started. The subsequent records indicate the changes brought about by continuous slow infusion with digitalis.

Rabbit—A typical result obtained in rabbits is indicated in the top records of Plate XII, fig 2. The record *a* was taken with the animal under urethane anaesthesia and before the digitalis infusion was started. In this and in the subsequent records of the electrocardiogram of the rabbit lead 2 only was used. An electrocardiogram was taken before the administration of the anaesthetic and differed in no way from that taken during the anaesthesia. The next record, *b*, was taken 21.5 minutes after infusion with digitalis was started and 16.8 c.c. of the solution had entered the vein. There is hardly any change in the electrocardiographic features with the exception that the *T* wave appears more pointed. As the infusion was continued, the depression of the *S-T* interval soon followed and was present in the record taken two and a half minutes later when further 2 c.c. of digitalis solution had entered the vein. This depression was progressive and resulted in a marked upward concavity of the *S-T* interval with a sharply-pointed *T* wave. As the infusion proceeded, the *P* wave disappeared and the electrocardiogram assumed an infarct type in which the *S* wave did not return to the base line. These features are brought out in the records *c* and *d* which were taken 39 and 42 minutes respectively after infusion with digitalis solution was started. The *P* wave is not visible in these records and the heart action is irregular, the auricles evidently fibrillating. In all these records, the rate of the heart is in the neighbourhood of 300 per minute. As further infusion proceeded, the ventricular action soon became more irregular and less frequent, the heart stopping about 8 minutes after the record *d* was taken.

Dog—Altogether four dogs were used and in these all the three standard leads were studied. The results obtained in one of the dogs is shown in Plate XII, fig 2. The dog, weighing 11 kilos, was anaesthetized by giving 20 c.c. of paraldehyde through the stomach tube. For the sake of convenience, only the results obtained in lead 2 are illustrated. In lead 1 the excursions were comparatively small and the alterations of the features with digitalis infusion were not so marked as in the other two leads. Lead 3 gave results almost identical with those of lead 2.

Plate XII, fig 2*e*, is the record taken while the animal was under the anaesthetic and before the digitalis infusion was started. It shows a prominent *P* wave, well-marked *R* and *S* waves and a slightly inverted *T* wave. The *S-T* interval has a convexity upwards. In the next record, *f*, which was taken 16 minutes afterwards when 40 c.c. to 45 c.c. of digitalis solution had entered the vein, a marked change has occurred. The *T* wave has become upright and pointed, and the *S-T* interval has no longer the upward convexity, it has become depressed. These features are even more marked in the next record, *g* which was obtained 17 minutes after *f* when 80 c.c. of digitalis solution had entered the vein. At this stage the animal's respirations had become rapid with jerky inspiration and expiration. The heart sounds had become very feeble. The last record in this animal is *h* which was taken 34 minutes after the previous one when 150 c.c. to 155 c.c. of digitalis solution had been introduced. The *P* wave has disappeared and the record has the appearance found in paroxysmal tachycardia of ventricular origin, though there is a possibility of auricular fibrillation with bundle-branch block. The animal died 6 minutes later.

Cat—Electrocardiograms were taken in cats in only two cases. Records obtained in one of them are shown in Plate XII, fig 2. The animal was a male cat weighing 4.2 kilos. It was anaesthetized with intraperitoneal injection of 4.2 g of urethane and later was given 3 c.c. of paraldehyde per stomach. Electrocardiograms with all the three standard leads were taken of which only lead 2 is shown. The record *i* was taken while the animal was under the anaesthetic before starting the digitalis infusion. The *T* wave is inverted. It was inverted in leads 1 and 3 also. It is not sure whether this be considered a normal feature in this animal or be due to a pathological condition of the heart or to the action of the anaesthetic. The next records *j*, *k* and *l* were taken when 23 c.c., 43 c.c. and 54 c.c. respectively of digitalis solution was introduced. The rate of infusion was approximately 1 c.c. per minute. A marked depression of *S-T* interval is noticeable and the *T* wave appears pointed upwards.

DISCUSSION

In the mammalian experiments the most constant feature of the action of digitalis is the depression of the *S-T* interval. This is most marked in the case of the rabbit but is also quite definite in the dog and the cat. In the rabbit an infarct type of electrocardiogram has developed. In no case however a *T* wave which was upright in the beginning has become inverted as the result of the action of digitalis. On the contrary, in certain cases, the *T* wave, which was inverted at the start, has become markedly upright after infusion with the digitalis solution. This feature is also seen in the case of the perfused frog heart, which is all the more remarkable as the mode of propagation of the impulse in the frog heart must evidently be different from that in the mammalian heart.

It seems, therefore, that the depression of the *S-T* interval is the initial and the most constant feature of the effect of digitalis on the electrocardiogram. Digitalis does not seem to cause inversion of the *T* wave. It has not done so in many acute experiments on animals. On the other hand, a reverse effect is sometimes seen, a *T* wave may become markedly upright as a result of the action of digitalis. In this connection the experiments of Brams and Gaberman (*loc cit*) are interesting as they show that on the healthy human heart also, as far as the *T* wave is concerned, the results are similar to those seen in acute experiments on animals.

SUMMARY

Electrocardiographic changes were studied on isolated frog's heart perfused with digitalis solution and also on the hearts of anaesthetized rabbits, cats and dogs, by intravenous infusion, in acute experiments of 1-20 tincture digitalis.

The initial and the most constant change in the electrocardiographic feature was found to be a depression in the *S-T* interval, followed later by an infarct type of *Q R S* complex. The *T* wave did not become inverted. On the contrary there was a tendency for the *T* wave to become more erect and pointed.

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STUDIES ON PEPTIC ULCER IN SOUTH INDIA

Part IV.

INCIDENCE OF PEPTIC ULCER IN INDIA WITH PARTICULAR REFERENCE TO SOUTH INDIA

BY

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(Work done under the Indian Research Fund Association)

(From the King Institute, Guindy, Madras)

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INTRODUCTION.

IN India, till not very long ago peptic ulcer was neither recognized nor looked for amongst Indians. Gastric and duodenal ulcers were considered rare. The pioneer work of Somervell, Bradfield and Pandalai, in South India, however, has made the surgeons in India 'ulcer conscious'. More cases are therefore being dealt with surgically throughout India and, consequently, the disease is now recognized as of quite frequent occurrence. No attempt at estimating the incidence of the disease had been made in the past. In the present communication extensive data, made available by this inquiry, are presented with a view to indicate the incidence and distribution of the disease in various provinces in British India. This is considered to be of special importance in view of the most serious infirmities caused by this disease, the great crippling effects on the individual and the prolonged convalescence and consequent loss of man-power to the state.

In the absence of a village-to-village survey, in a vast country such as India with lack of easy and cheap means of transport between widely scattered villages and its ignorant and poverty-stricken population, even if all sources of medical relief are included, a correct estimation of the incidence of this disease is not possible. The medical relief centres where adequate facilities for diagnosis and treatment of this disease are available are very few and far between and it is not

a matter of mere conjecture that a large number of cases of this disease is missed. It is thus evident that, whatever the estimates, the actual incidence of the disease is likely to be much higher.

MATERIAL AND METHODS

Through the courtesy of the Director-General, Indian Medical Service, a proforma was circulated to all the provinces of British India and statistics were thus collected. Through the administrative heads of the medical departments in each province, original returns from hospitals and dispensaries were obtained. All Government, Government-aided, Local Fund and other charitable hospitals and dispensaries were included. By so including all possible sources it was hoped that a fairly accurate estimation of the incidence of the disease might be obtained particularly when total provincial figures are considered in each province, because, as a rule, patients do not travel from one province to another for reasons of health.

It was recognized that such returns, particularly from out-patient dispensaries and small hospitals in outlandish places with their poor equipment and excessive attendance, could not be absolutely relied upon as far as the diagnosis of peptic ulcer was concerned. Again, owing to the lack of scientific accuracy on the part of the majority of the medical profession, and the absence of any uniformity in nomenclature, a duodenal ulcer may be classified as such or as peptic ulcer, pyloric obstruction or even gastric ulcer. Further, although the disease was well recognized in the South it was not so in the North—as will be shown subsequently—a large majority of the so-called 'dyspepsias' may be duodenal ulcers. It was, therefore, deemed necessary to ask for returns of the total number of cases of the 'disease of the stomach' treated at various institutions sub-classified under the heading indigestion, dyspepsia, gastritis, peptic ulcer, gastric ulcer, duodenal ulcer, pyloric obstruction and jejunal ulcer. Figures for the calendar year 1939 were thus collected. In addition, annual reports of the working of the hospitals and dispensaries in various provinces and Indian States have been studied to determine the variations in the incidence of gastric disorders, number of operations on the stomach and various other pertinent data.

It must also be pointed out that the figures do not represent the number of patients. A patient on his first application for treatment is entered as a new case till he stops coming to the hospital. At all subsequent applications by the same patient for the same complaint he is again shown as a new case. Thus, as often happens in the case of 'stomach cases', one patient may be shown on two or three occasions in the returns. This error, of course, is common to other diseases as well, and takes place in all the provinces surveyed and, as such, is disregarded for the purposes of this communication.

DISEASES OF THE STOMACH

Subject to the limitations enumerated above, the data of the diseases of the stomach are of considerable interest. Surprisingly enough, although peptic ulcer is

reported to be rare in the North of India, gastric disorders occur throughout India and show very little variation from year to year as is shown in Table I compiled for the five-year period (1933-1937) Only the major provinces in British India are included .

TABLE I

Diseases of the stomach for the five-year period (1933-1937)

Province	Maximum	Minimum	Average
Madras	464,437	338,078	388,266
Bihar and Orissa	202,128	163,922	176,710
N W F P	95,850	68,816	84,806
Assam	74,654	54,768	69,660
C P and Berar	93,468	85,281	91,140
Bombay	127,457	46,033	93,700
Bengal	260,414	242,427	251,310
Punjab	645,270	563,518	607,508
United Provinces			264,659*

* Only 1937 figures available

The figures for the province of Bombay are exceptional. It is worth noting, however, that whereas figures for 1933, 1934 and 1935 showed no variation, the number suddenly dropped from 127,457 to 46,933 in 1936 and to 54,811 in 1937 respectively. This was due presumably to the separation of Sind from Bombay

as a separate province The number of operations on the stomach during these two years, however, increased from 45 in 1933 to 115 and 111 in 1936 and 1937 respectively, i.e. an increase of almost 150 per cent The significance of this is discussed later The incidence of the diseases of the stomach in various provinces of British India are shown in Table II —

TABLE II

Name of the province	Population (1931 Census)	Average number of patients treated, indoor and outdoor	Average number of cases of the diseases of stomach	Percentage	Diseases of the stomach per 10,000
Bengal	51,087,070	1,040,712	251,319	3.11	49
United Provinces	49,614,833	8,407,528	264,650	3.14	53
Madras	46,740,107	16,609,885	388,266	2.31	83
Bihar and Orissa	42,329,583	5,671,978	178,710	3.15	42
Bombay	26,398,997	3,154,661	93,709	2.94	50
Punjab	23,580,852	14,256,757	607,508	4.25	258
Central Provinces	17,290,937	3,154,661	93,709	2.94	35
Assam	9,247,857	2,168,618	69,669	3.19	75
N W F P	4,684,364	2,002,124	84,274	4.10	180

It would appear that of the total attendance at hospitals and dispensaries for the diseases of the stomach varied from 2.31 to 4.25 per cent, and incidence of 35 to 258 per 10,000 of population Whereas the Punjab and the North-West Frontier Province showed the highest incidence and Madras a little more than

the Central and the United Provinces, the province of Bihar and Orissa was the lowest

The 1939 figures, obtained especially for the purpose of the present investigation, show the distribution of the diseases of the stomach in the 11 provinces including the two new ones, namely, the provinces of Sind and Bihar. These are shown in Table III —

TABLE III

Name of the province	Population (1931 Census)	Diseases of the stomach	Incidence per 10 000 population
Bengal	51,087,338	363,349	71
United Provinces	49 614 833	494,785	100
Madras	46,740 107	863,632	185
Bombay	26,398,997	196,744	75
Bihar	23 676 028	389,731	165
Punjab	23,580,852	782,471	332
Orissa	18 653 555	128,141	69
Central Provinces	17 990 937	234,313	130
Assam	9,247,857	102,937	111
N W F P	4 684 364	146,406	290
Sind	3,887,070	86,337	222

It must be noted that in each province these figures are considerably higher as compared with those in Table II, which are average figures, presumably due to (a) greater care with which the statistics have been collected by including all sources, (b) increase in the population in recent years and (c) increase in the number of hospitals and dispensaries throughout the country where more people are now being

attended to. It should be observed that the incidence of the diseases of the stomach during 1939 was 69 to 332 per 10,000 population.

The conditions prevailing in South India, described elsewhere (Dogra, 1940a), are notable in each province, namely (i) The largest number of cases is reported from capital city hospitals due to facilities available for adequate treatment and diagnosis and consequent reputations of such hospitals. (ii) At these centres the number of operations for gastro-duodenal ulcer is high and the cases treated for dyspepsia low. (iii) In out-stations the cases returned as indigestion, dyspepsia and gastritis are considerably higher. It is suggested that a large number of ulcer cases is missed due to lack of facilities for diagnosis in these out-station hospitals and dispensaries, as they are labelled as dyspepsias and treated as out-patients.

It is interesting to record here that wherever the number of gastro-duodenal ulcers diagnosed and surgically treated is low the number of dyspepsia cases is high. This is particularly striking in the two Indian States in South India where this question was studied.

In Travancore, with a total population of just over five millions, an average of 300 cases of gastro-duodenal ulcer are operated upon every year and about 15,000 cases treated for dyspepsia at the various hospitals and dispensaries throughout the state. In Mysore, on the other hand, with a total population of just over six millions, about 50 cases of gastro-duodenal ulcer are operated upon and 150,000 treated for dyspepsia. Various environmental conditions, economic state, etc. of gastro-duodenal ulcer cases prevailing in these two states are similar as already described (Dogra, 1940a). It is inferred, therefore, that the number of dyspepsia cases is inversely proportional to the number of gastric cases treated surgically in any given area.

PEPTIC ULCER CASES

(a) *All cases*—The term diseases of the stomach includes a multitude of conditions varying from gastritis to an ulcer. Obviously the number of actual cases of peptic ulcer would be much less.

Experience in South India showed that gastric ulcer was a rare disease and although gastric carcinoma was occasionally met with, the vast majority of 'gastric cases' were those of duodenal ulcer occurring alone or associated with an ulcer in the pyloric antrum. Clinical diagnosis, where not confirmed at operation, was often difficult and it was quite a common thing to see a duodenal ulcer labelled as a 'gastric ulcer'. The relative proportion of duodenal and gastric ulcer was 30 to 1 amongst cases operated on. All the cases of pyloric obstruction, except when due to carcinoma of the stomach, were cases of duodenal ulcer leading to stenosis of the duodenal and pyloric regions (Dogra, 1940b). It may, therefore, be considered that figures returned under peptic ulcer, gastric ulcer, duodenal ulcer and pyloric obstruction represent primarily figures for peptic ulcer. Table IV shows the incidence of peptic ulcer, so considered, in the various provinces in British India. It must, however, be borne in mind that these figures do not represent the total number

of peptic ulcer cases because a fair proportion of such cases are missed and labelled as dyspepsia, gastritis and indigestion, as pointed out above

TABLE IV
(1939 figures)

Name of the province	Population (1931 Census)	Cases of peptic ulcer	Incidence per 100,000 population
Bengal	51 087 338	16 976	33
United Provinces	49 614 833	4 460	11
Madras	46 740 107	57 397	143
Bombay	26 398 997	2 099	7
Bihar	23 676 028	8 851	37
Punjab	23 580,852	2 131	9
Orissa	18 653 555	4 132	29
Central Provinces	17 990 937	2 327	12
Assam	9,247 857	926	10
N W F P	4 684 364	421	1
Sind	3,887 070	315	8

These figures are interesting in that Madras shows the highest incidence with 143 Bihar 37, Bengal 33, Orissa 22 Central Provinces 12 per 100,000 population, the Punjab and the North-West Frontier Province being the lowest, namely 9 and 1 respectively. The difference in the North and the South thus appears to be that of 1 15 and not 1 58 (McCarrison, 1921) or 1 600 (Somervell and Orr, 1936)

(b) *Cases operated on*—The number of operations on the stomach carried out in the whole of India during the year 1933–1937 is shown in Table V. The figures include gastroenterostomy, partial gastrectomy and other operations carried out

for gastro-duodenal ulcer and pyloric obstruction, except secondary operations on the stomach and lavage. Lavage is returned as an operation on the stomach in the statement 'E' of the annual reports from the hospitals and dispensaries in the various provinces in India and is excluded here for obvious reasons.

TABLE V
Operations on the stomach for the years 1933-1937

Province	1933	1934	1935	1936	1937
Madras	1,067	1,031	1,080	966	1,020
United Provinces					48
N -W F P	2	5		7	5
Assam	1				
C P and Berar	4	11	10	6	30
Bombay	48	32	65	115	111
Bengal	159	110	100	136	84
Sind					10
Bihar					18
Orissa				17	13
Punjab	1	6	8	4	25
TOTALS	1,282	1,195	1,263	1,251	1,364

It is apparent that whereas the relative incidence of peptic ulcer in the North (Punjab) and South (Madras) of India is roughly 1 : 15, the number of cases in which operative treatment is given shows a marked difference. This difference has been misleading to previous workers who estimated incidence of peptic ulcer from figures of surgical operations in different provinces.

Surgical treatment is not applied for or even agreed to by the majority of Indian villagers. Except for a few centres adequate facilities for such surgical operations as gastroenterostomy and gastrectomy are not available. Again, only recently, the medical profession has recognized the frequency with which peptic ulcer occurs in this country and the efficacy of surgical treatment in this disease. In certain parts the medical profession is still not 'ulcer conscious'. The state of affairs is comparable to what pertained in South India in 1922 when Bradfield (1927) started his work in Madras.

Study of hospital statistics at Madras Government General Hospital, and London Mission Hospital, Neyyoor, for several years past showed that the number

of operations on the stomach suddenly increased several hundred per cent, subsequent to the arrival and pioneer work of the two great surgeons in South India, namely Bradfield in Madras, and Somervell in Neyyoor. Table VI substantiates this statement. Although the figures for 33 years were studied, only a few are mentioned in this table —

TABLE VI
Operations on the stomach

	1913	1919	1922	1927	1933	1937
Madras (G. G. H.)	13	6	129	362	507	348
Neyyoor (L. M. H.)	6	37	47	227	333	253

Considering the 20-year period (1913-33) it would appear that the number of cases operated upon for peptic ulcer at the two centres in South India increased 39 and 56 times respectively. This obviously is not the result of increase in the incidence of the disease in South India during this period, but directly to the fact that these surgeons on arriving in 1922 at their respective hospitals recognized the disease and successfully carried out surgical treatment of a large number of cases which previously would have been labelled as 'dyspepsia', 'Malabar dyspepsia', 'coco-nut dyspepsia', etc., etc.

This increase in the surgical treatment of peptic ulcer as indicated by the number of operations on the stomach has also been evident in other provinces in recent years, e.g. (i) Bombay, where the number has increased from 45 in 1933 to 111 in 1937, i.e. nearly 150 per cent increase, (ii) Punjab, where the number has increased from 1 in 1933 to 25 in 1937. The same is true of other provinces as shown in Table V above.

It must also be borne in mind that the cases operated upon at any one hospital are not necessarily those from the areas in which the hospital is situated. For example, at Neyyoor during 1933, of the 227 cases operated upon, there was only one case from Neyyoor itself. At the Government General Hospital, Madras, of the 258 cases investigated, only 54 were cases from Madras itself and the rest came from out-stations.

From what has been said above, it is clear that the number of operations performed at a particular centre in any particular part of the country does not indicate the incidence of the disease in that particular locality. Consequently, increase in the number of operations cannot be considered indicative of the increase in the prevalence of the disease in the area under consideration.

DISTRIBUTION OF THE CASES OF PEPTIC ULCER

It is apparent that peptic ulcer occurs throughout India. It is more prevalent in the South. The exact extent is not possible to determine, but, subject to the limitations enumerated above, the relative incidence in different parts of the country is of considerable interest. The Map of India shows the incidence of peptic ulcer in different provinces.

The greater incidence of peptic ulcer in Madras, Bihar, Bengal and Orissa is noteworthy in contrast with the rest of India. The significance of this difference is not apparent. It may, however, be stated that in all these provinces rice is the staple diet and the common people consume a diet which is high in carbohydrate and low in protein content, whereas the conditions in the rest of the country are different. Again, comparatively the ryot is very much poorer in these provinces. The ætiological bearing of these facts will be discussed in another communication on the pathology and ætiology of peptic ulcer in India.

SUMMARY AND CONCLUSIONS

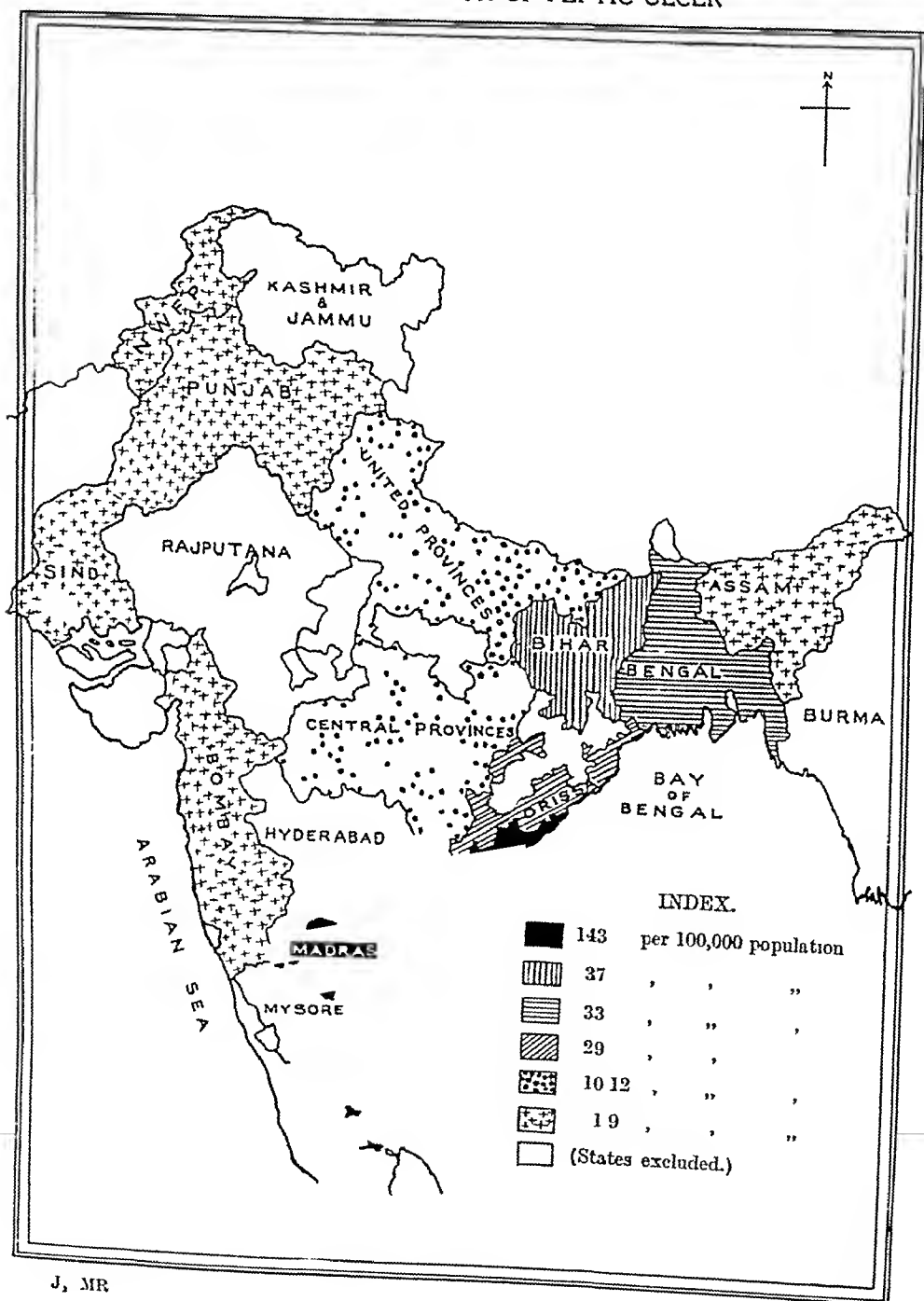
Statistics from different parts of India regarding diseases of the stomach are presented and incidence of peptic ulcer in India estimated. From all over British India 5,674 hospitals and dispensaries responded to the request for data. Limitations of these statistics are noted. A study of the triennial reports on the working of the hospitals and dispensaries in various provinces was made. An analysis of data for a five-year period (1933-37) is recorded.

The data regarding the number of surgical operations on the stomach from various provinces during several years past are reported. The unreliability of the number of operations on the stomach as an indication of the incidence of peptic ulcer in any particular locality is emphasized. It is suggested that, although true estimation of the extent of this disease is not possible, a rough estimate may be obtained by including cases under the categories of peptic ulcer, gastric ulcer, duodenal ulcer and pyloric obstruction. From our experience of cases operated on in South India where the vast majority (about 97 per cent) of 'gastric cases' are duodenal ulcers, it is suggested that such combined figures would be the nearest to the actual number of cases of this disease in India.

The number of operations on the stomach varies considerably in each province and whereas in the province of Madras there seems no marked variation in recent years, there is considerable increase in other provinces. This is ascribed to the medical profession becoming 'ulcer conscious', a state of affairs that occurred very dramatically in South India in 1922 when Bradfield and Somervell arrived and began their pioneer work on gastric surgery in this country.

The distribution of the cases of peptic ulcer is shown. The greater incidence of ulcer in provinces where a high carbohydrate and low protein diet is prevalent is considered of importance with regard to ætiology of this disease in India.

MAP OF INDIA SHOWING DISTRIBUTION OF PEPTIC ULCER



The following conclusions are drawn —

(i) Diseases of the stomach are common throughout India constituting 2 to 4 per cent of the total attendance at the hospitals and dispensaries. The 1939 figures in which were included all sources and which, possibly, were collected more carefully show an incidence of 690 to 3,320 per 100,000 population.

(ii) Peptic ulcer occurs all over India.

(iii) Incidence of peptic ulcer varies from 9 to 143 per 100,000 population. The highest being 143 in the province of Madras. The provinces of Bihar, Bengal and Orissa show an incidence of 37, 33 and 22 per 100,000 population. The rest of the country shows a uniform distribution with an incidence about 10 per 100,000 population. Subject to the limitations already recorded, it would appear that peptic ulcer is 15 times more common in Madras (South) than in the Punjab (North).

ACKNOWLEDGMENTS

This work has been made possible through the courtesy of the Director-General, Indian Medical Service, under whose direction administrative heads of medical organizations in various provinces collected these statistics. My thanks are due to the innumerable medical officers in-charge of hospitals and dispensaries throughout the length and breadth of India who actually laboured to collect and submit the material. Thanks are also due to the Director, King Institute, Guindy, for his encouragement, to Mr P R Seshadri, for his invaluable clerical assistance, and to the Indian Research Fund Association for their financial aid.

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OCCURRENCE OF *WUCHERERIA BANCROFTI* INFECTION IN A RURAL AREA

BY

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IN a previous communication (Iyengar, 1939a) it was mentioned that the distribution of *Wuchereria bancrofti* infection in India was characteristically urban. The urban distribution of this infection and its comparative absence in rural areas were attributed to the fact that *Culex fatigans*, the chief transmitter of the infection, occurred mainly in urban areas.

It was also remarked that malaria and *W. bancrofti* infection did not ordinarily occur together, not because of any antagonism between the two infections, but owing to the fact that they had 'different spatial distributions, inasmuch as the epidemiological factors favouring the prevalence of the one are adverse to the prevalence of the other' (Iyengar, 1938). The occurrence of endemic *W. bancrofti* infection was associated with a high incidence of stagnant waters heavily contaminated with decaying organic matter of the kind favourable for the breeding of *Culex fatigans*. Such extensive organic pollution of the water collections was inimical to the development of vectors of malarial infection and consequently malaria rarely occurred in an area where *W. bancrofti* infection was endemic.

Recently, endemic *W. bancrofti* infection was observed to occur in a typically rural area in Bengal. During a malaria investigation in Illambazar thana (Birbhum district), cases of endemic filariasis were observed in several villages in the area*. In two of these villages, namely Bharatpur and Nabagram, an investigation was made to determine the incidence and type of filarial infection. The findings of

* Cases of filariasis were observed in six villages in this thana, namely Nilagarh, Narayanpur, Gangapur, Barupur, Bharatpur and Nabagram.

an examination of samples of the populations of the two villages are given below —

Village	Number of persons examined	Number with microfilaria in peripheral blood at night	Filarial infection rate
Bharatpur	51	9	17.6
Nabagram	60	10	16.7

Out of 111 persons examined in the two villages 19 showed microfilaria in peripheral blood at night, an infection rate of 17.1 per cent. The microfilaria were determined as *W. bancrofti* (Cobbold). Six persons out of 111 examined showed external manifestations of filariasis, i.e. five showed elephantiasis of the leg and one showed elephantiasis of the leg and scrotum, these cases were negative for microfilaria in peripheral blood.

INCIDENCE OF MALARIA

In all the villages with cases of filariasis, a high incidence of malaria was noticed, the spleen rates among children varying between 42 and 84. In Bharatpur and Nabagram the spleen rates were 56.0 and 84.2 per cent respectively. The transmitter of malarial infection in Illambazar thana was *Anopheles philippinensis* which showed natural infection with malaria parasites to the extent of 7.2 per cent (Iyengar, 1939b). The fact that endemic malaria occurred concomitantly with filarial infection would lend support to the view previously expressed that there could be no antagonism between the occurrence of filarial infection and of malarial infection in a community.

TRANSMITTERS OF FILARIAL INFECTION

Mosquitoes caught from these villages were examined for filarial infection. The following gives the species and number of specimens examined: *Culex vishnu* 18, *Eicallia hybrida* 7, *Anopheles annularis* 7, *A. hyrcanus* var. *negerrimus* 9, *A. pallidus* 5, *A. varuna* 2 and *A. philippinensis* 13. The results were negative except in regard to *Anopheles philippinensis* in which two out of 13 specimens examined showed fully-developed filaria larvæ. The measurements and morphology of these larvæ corresponded to those of full-grown larvæ of *W. bancrofti* in experimentally infected *Culex fatigans*. In the neighbouring village of Barupur which also had endemic cases of filariasis, six out of 104 specimens of *A. philippinensis* examined showed well-developed filaria larvæ. These observations indicated that *W. bancrofti* infection in this area was transmitted by *A. philippinensis* which was also the vector of malarial infection.

A unique specimen of *A philippinensis* showed both filarial and malarial infections. This specimen which was collected from the village of Nabagram showed the salivary glands packed with innumerable sporozoites and at the same time the labium and thorax contained several well-developed filaria larvæ. This would indicate that the same mosquito could transmit both malarial and filarial infections at the same time.

Contrary to what obtains in other parts of India with endemic *W bancrofti* infection, where the vector is the urban mosquito *Culex fatigans*, in the villages of Illambazar thana the infection is transmitted by the rural mosquito *A philippinensis*. Similar conditions are reported to occur in the Dutch East-Indies and New Guinea where *W bancrofti* infection is essentially rural in distribution and the infection is transmitted by rural species of mosquitoes.

SUMMARY

Wuchereria bancrofti infection which was known to have an urban distribution in India was recently found to occur in a rural area in Birbhum district (Bengal). The filarial infection rate in two villages examined was 17 per cent. Cases of elephantiasis of the leg and scrotum were also observed to occur. These villages showed a high incidence of malaria indicating that the presence of malarial infection in the population was not antagonistic to the occurrence of filarial infection.

The transmitter of malarial infection as well as of filarial infection in these villages was found to be *Anopheles philippinensis*. In one specimen of *A philippinensis*, both malarial and filarial infections were observed, the salivary glands showed a heavy infection with sporozoites, while within the labium and thorax were many full-grown larvæ of *W bancrofti*.

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A PRESERVING MEDIUM FOR THE TRANSMISSION OF SPECIMENS FOR THE ISOLATION OF *VIBRIO CHOLERÆ*

BY

K V VENKATRAMAN,

Ind Jour Med Res, 29, 4, October, 1941

CORRIGENDUM

In paper entitled 'The Helminth Parasites of Dogs in Calcutta and their Bearing on Human Parasitology' by P A Maplestone and N V Bhaduri, *Ind Jour Med Res*, Volume 23, No 2 (October 1940), page 595, a trematode is listed as '*Troglorematidæ* (new genus?)' It should be named '*Prosthodendrium orimagnosum* (Bhalerao, 1925) Dollfus family *LECITHODENDRIIDÆ*'

The authors are indebted to G D Bhalerao D Sc, Ph D, for drawing their attention to this error—P A M and N V B

its isolation difficult or impossible Greig (1913) found the average life of the vibrio in rice-water stools stored in a dark cupboard at room temperature was 7 to 8 days in cool weather and 2 days in hot weather Soda (1936), investigating the question of the delay within which stools should be examined in the search for *V cholerae*, found that the vibrios die within 3 hours at body temperature, though they may remain viable for a longer time at room temperature and could be isolated in 2 to 8 days when stored in the ice-box He arrived at the conclusion that the material for examination should be collected within less than 24 hours previously and stored at a low temperature

Under the conditions of public health laboratory practice obtaining in this province, it is often necessary to examine specimens which take a considerable time in transit, and it is not always possible to hold them at a low temperature during the period There is need, therefore, for a preservative in which the cholera vibrio, if present in the stool at the time of collection, will remain viable till the specimens can conveniently be taken up for examination in the laboratory It is also essential

that the preserving fluid should inhibit the growth of other intestinal bacteria normally present in stools, including the so-called 'non-agglutinable' vibrios, if the recovery of true *V. cholerae* is to be facilitated. The preserving solutions that are in ordinary use for the transmission of dysenteric and typhoid stools, such as 30 per cent glycerine saline, were found not so suitable for cholera stools on account of their lethal action on *V. cholerae*. A number of dyes and other bacteriostatic agents have been tried, none of which proved satisfactory. Boric acid, which enters into the composition of highly alkaline buffers, seemed to be less inhibitory to *V. cholerae* in dilutions in which it inhibits the growth of *Bact. coli* and *Bact. aerogenes*.

Salt requirements—Read, Singh, Seal and Bose (1939) refer to the work of previous investigators who found that the presence of salt facilitates the survival of *V. cholerae* and record their own finding that both organic matter and salt are essential for the growth and survival of the cholera vibrio. Genevray and Bruneau (1938) claim that 3 per cent dried sea-salt in peptone water promotes the preferential growth of *V. cholerae* in the presence of *B. pyocyaneus* and *B. proteus*. Our own observations favour 2 per cent sea-salt as an optimum concentration conducive to prolonged viability of *V. cholerae* in the presence of other bacteria. We could recover the cholera vibrio after a delay of 198 days from a 2 per cent sea-salt solution inoculated with a mixture of *V. cholerae*, a non-mannose fermenting non-agglutinable vibrio and *Bact. aerogenes*.

Hydrogen-ion concentration—The value of high alkalinity in promoting the preferential growth of the cholera vibrio in the presence of *coliform* and other organisms is well recognized. Read *et al.* (*loc. cit.*) record that *V. cholerae* in pure culture multiplies best in the pH range 6 to 9 and that good multiplication could be secured at pH 9.4. In our experiments on survival of *V. cholerae*, we found a pH of 9.2 most suitable.

Boric acid tolerance of *V. cholerae* and coliform organisms—To demonstrate the difference in the boric acid tolerance of *V. cholerae* and *coliform* organisms three sets of tubes of peptone water (peptone 1 per cent, NaCl 0.5 per cent, pH 8.4), containing increasing amounts of boric acid, were inoculated with *V. cholerae*, *Bact. coli* and *Bact. aerogenes*, respectively. The size of the inoculum was so adjusted that each tube received approximately 100 organisms. Immediately after inoculation, and again at intervals of 4, 8, 24 and 48 hours, a standard loopful of the culture from each tube was spread on agar, incubated overnight and the number of colonies on each plate counted. It was seen that, while boric acid inhibits to some extent the growth of all three, *V. cholerae* survives a concentration of up to 1.5 per cent, while both *Bact. coli* and *Bact. aerogenes* are completely inhibited by 0.6 per cent and, to a considerable extent, by even 0.3 per cent. The difference was even more marked when the organisms were held in salt solution containing 1/50,000 peptone, 0.3 per cent boric acid was sufficient to completely inhibit *Bact. aerogenes* and *Bact. coli*. This differential inhibition was, however, lost with heavy inocula of the size of 1 c.c. of a 24-hour broth culture.

The preserving solution—Based on the considerations mentioned above, a buffered sea-salt solution of pH 9.2 was prepared from the standard mixtures of

Clark and Lubs (1916, quoted by Clark, 1923) and its value as a preserving medium investigated. The solution was prepared as follows: 12.405 g boric acid (H_3BO_3) and 14.912 g potassium chloride (KCl) are dissolved in about 800 c.c. of hot distilled water, the solution cooled and made up to 1 litre. From this stock solution, 250 c.c. are taken, mixed with 133.5 c.c. of M/5 NaOH and the whole made up to a litre. Twenty grammes dried sea-salt (common salt from the bazaar serves equally well) are dissolved and the buffered saline filtered through paper, dispensed in 10-c.c. quantities in 1-oz. screw-capped bottles and sterilized in the autoclave. The sterilized buffer has a pH of 9.2 and is found to maintain the same pH for months. The collecting outfit includes, for convenience, a small aluminium spoon which will hold about 1 g. to 3 g. of stool depending on the consistency. In use, a spoonful of the stool specimen is well mixed in the buffer which is then mailed.

Growth and survival of V. cholerae in specimens of artificially infected stool preserved in the medium—Normal human stool whose reaction has been adjusted to a pH of 9.2 by the addition of alkali, was suspended in buffered saline prepared in the manner described above, and the suspension strained through muslin. This uniform stool suspension was distributed in 10-c.c. quantities in a number of sterile 1-oz. screw-capped bottles. Each bottle received an inoculum of 1 c.c. of a suitable dilution (10^{-6} to 10^{-9} in different experiments) of a 24-hour broth culture of a laboratory strain of *V. cholerae*, containing approximately from 10 to 10,000 (in different experiments) vibrios as determined by culture of decimal dilutions of the inoculum. The specimens were held at room temperature and each day, various quantities of the preserved specimens were enriched in Read's modification of Wilson and Blair's medium (Read, 1939) and subsequently plated on Aronson and agar media. It was seen that there was only a slight initial multiplication (in the region of a tenfold increase), but the vibrios remained viable for as long as 62 days. Recently isolated strains of *V. cholerae* were used in other experiments with substantially similar results.

Survival of V. cholerae in a stool from a proved case, preserved in the medium—A set of 24 specimens was taken in the preservative from a bacteriologically positive case on the first day of illness. These were examined at intervals, after preliminary enrichment in mannose-bismuth-sulphite medium. It was found possible to isolate *V. cholerae* up to 92 days after collection, when the specimens were exhausted. The pH of the specimens remained at 9.2 at which it was originally adjusted.

A field trial of the preservative—An opportunity to test the practical value of the preserving solution under natural conditions was afforded by an outbreak of cholera in the Northern Circars. Two sets of specimens of stools were collected in the preservative from clinically typical cases of cholera. One set was examined immediately on the spot, while the other was mailed to the laboratories at Tanjore, taking 4 to 7 days in transit, and occasionally longer. Both in the field and in the laboratory, platings were made after preliminary enrichment in mannose-bismuth-sulphite medium. Sixty-six specimens, including a specimen of vomit, were taken from 60 cases. *V. cholerae* was isolated from 64 specimens in the field, and

from 60 in the laboratory. No case was missed. In two instances, *V. cholerae* was isolated from mailed specimens, while the immediate examination of specimens taken in the field proved negative. We had the impression that failure to isolate *V. cholerae* occurred in those instances where an excess of stool had been added to the preservative causing a drop in the pH.

The preserving solution was also utilized in the examination of 135 healthy contacts of 23 cases. Three hundred and seventy-three specimens were obtained from 135 contacts and the cholera vibrio recovered from 8. One of these appeared to have been incubating the disease as he subsequently passed through a mild attack but the other 7 remained healthy. These observations were not controlled by attempts at isolation by immediate examination, but the percentage of healthy contacts detected passing *V. cholerae* is considered sufficiently high to warrant the belief that not many had been missed.

SUMMARY

A preserving medium for the transmission of specimens for the isolation of *V. cholerae* is described. From specimens of infected stool held in the medium, *V. cholerae* could be recovered up to 92 days after collection. The results of a trial of the preservative in the examination of specimens in an actual outbreak are given.

ACKNOWLEDGMENTS

Our thanks are due to the District Health Officers of Vizagapatam, West Godavari and South Arcot, for their co-operation in the collection of the field specimens and for the facilities they afforded us in the examination of cases.

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ON THE FETAL INFECTION BY
L ICTEROHEMORRHAGIÆ
IN A RAT

BY

M N LAHIRI

(*From the Haffkine Institute, Parel, Bombay*)

[Received for publication, May 12, 1941]

It is established that rat is the carrier of pathogenic leptospiræ, but no definite observations have been made as to how the infection is maintained in the rat population. Since leptospiræ are excreted in urine of the infected rat, it has been suggested that the infection occurs through the ingestion of food contaminated with such urine. Possibly also leptospiræ may gain entrance into the body through the infected material coming in contact with the skin with open wounds. The observations to be described in this paper suggest still another possible mode of transmission, e.g. transmission of infection from the mother to the offspring through the placenta.

In the literature there is no mention of placental transmission of infection from mother to offspring in the rat though there are a few experimental observations on such transmission in the guinea-pig. In the case of guinea-pig Costa and Troisier (1916) were the first to report the penetration of leptospiræ through placenta as shown by the transmission of the disease by inoculation of the amniotic fluid. To this Buchanan (1927) put forth objections on the ground that the presence of infected blood in such circumstances is rather difficult to exclude. Takagi (1927) detected spirochaetes in the placentas of guinea-pigs infected with Weil's disease but the organisms could not be found in the foetuses. It was observed that the spirochaetes had been taken up by the ciliated cells and other tissues on the outside of the embryo which seem to protect the foetus from invasion from the maternal body. The transmission of leptospiræ from mother to foetus was established by Saenz (1929). This worker inoculated a pregnant guinea-pig with leptospiræ and five days later removed both the placenta and the foetus. The guinea-pig inoculated with the emulsion of the foetus died seven days later, whilst the second animal inoculated with the placenta died of the same infection after thirteen days. Another pregnant guinea-pig was infected six days before the birth of two young ones in

both of which leptospiræ were detected. More recently, Das Gupta (1939) undertook a series of experiments on the guinea-pig during various stages of pregnancy and advanced definite evidence of transmission of leptospiral infection from mother to the foetus. Further, the presence of the organism was demonstrated in the sections of the foetal liver. These observations, therefore, confirmed Saenz's findings.

During the course of investigation on the incidence of leptospiral infection in the rat population of the city of Bombay a rat of the species *Rattus norvegicus* in a fairly advanced state of pregnancy was trapped. On examination under dark-ground illumination the kidney emulsion of this animal showed the presence of leptospiræ. This chance presence of infection in a pregnant animal permitted of the following investigation —

The uterus of the rat was removed aseptically and six well-developed live foetuses were recovered. In the removal of the foetuses care was taken to avoid contamination with maternal fluids. Similarly, in order to eliminate any possible contamination from external sources, each foetus was immersed in a bowl of strong lysol solution for five minutes and then washed in several changes of sterile saline. The kidneys of each foetus were removed under aseptic precautions but owing to the paucity of young guinea-pigs saline emulsions of the kidneys of three foetuses were pooled together. Two cubic centimetres from each of the two mixtures thus made were injected intraperitoneally into separate guinea-pigs. On examining the mixtures under dark-ground illumination before inoculation leptospiræ were seen in only one of them. Two c.c. of emulsion of kidneys of the mother rat were also similarly inoculated into a young guinea-pig of about 180 grammes in weight. On the third day after inoculation the peritoneal fluid of the guinea-pig showed few leptospiræ under dark-ground illumination and on the seventh day after inoculation this animal died. On post-mortem examination jaundice and typical flecks of hæmorrhage all over the lungs, the two pathognomic signs of the infection in guinea-pigs, were noticed. The emulsion of the liver showed numerous leptospiræ and a second guinea-pig was inoculated with this emulsion. From this second animal the strain was finally isolated. The culture of the heart's blood of this animal yielded a luxuriant growth in Vervoot's medium.

The two guinea-pigs which received the pooled emulsions of the kidneys of the foetuses died respectively on the eighth and tenth day after inoculation. Post-mortem examinations presented the characteristic signs of leptospiral infection. A pure growth of leptospiræ was obtained from the culture of the heart's blood of both these animals. The three strains thus isolated from mother and the offspring were found to be serologically identical. The above observation is, therefore, a clear indication of natural infection of rats through placenta.

The question arises whether the embryonic rat infected in the uterus through placenta would survive after birth. On this point, however, we have no direct evidence. From the fact that the foetuses were all alive even at such an advanced stage of pregnancy it is very likely that they could have been born alive and would have developed to maturity. They would thus have acted as reservoirs of infection. This is further supported by the fact that there is no experimental evidence in

favour of *L icterohæmorrhagiæ* being pathogenic to rats Definite experimental studies in rats are, however, needed to clarify the point

SUMMARY

Intra-uterine infection of the foetuses of a rat through the placenta is recorded

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A PEPTIC DIGEST BROTH FOR THE FORMATION OF *CLOSTRIDIUM TETANI* TOXIN

BY

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AND

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[Received for publication June 30, 1941]

A REVIEW of present literature will show that Witte or Berna peptones are generally used for the production of tetanus toxin. Prevot and Boersma (1939) and Ramon (1939) have shown that beef protein suitably treated with a protease can also serve as a suitable medium. Presence of the earlier cleavage products of protein in the broth has been thus found to be essential for the production of this toxin.

While studying the degradation products of peptic digestion of veal, it has been observed by Sen (1941) that a good medium for potent tetanus toxin production can be obtained by incorporating veal infusion with veal digest in suitable proportion. Working under conditions noted below the toxin is found to possess good antigenic value.

PREPARATION OF THE DIGEST

Minced fat-free veal (1.5 kg) was mixed with distilled water (3 litres) and pH adjusted to about 1.6 to 1.8 with hydrochloric acid. Pepsin, previously standardized for its proteolytic activity in terms of haemoglobin units (Anson, 1938), was then added (about 2.4 haemoglobin units per 1.5 kg of veal) and the whole was incubated at 37°C for 96 hours.

ADJUSTMENT OF THE INFUSION

Veal infusion was made according to Wadsworth (1939) with slight modifications (water and veal being in equal proportion). The digest and infusion were then

mixed and the mixture brought to pH 4.8 (glass electrode) with 40 per cent caustic soda solution and kept in a water-bath at 70°C for 30 minutes so as to precipitate the metaproteins. The reaction of the broth was finally brought to pH 7.4. The proportion of the digest and the infusion in the mixture was such as to afford a broth of composition approximating the figures in the table. Amount of sodium chloride formed in the broth was estimated and the quantity required to give a final strength of 0.5 per cent was added.

Composition of the broth

	Per cent
Total nitrogen	0.599
Metaprotein nitrogen	0.008
Primary proteose nitrogen	0.218
Secondary proteose nitrogen	0.175
Amino nitrogen (van Slyke)	0.075
Peptone and residual nitrogen	0.130
Total solid (including 0.5 per cent NaCl)	5.06
Tyrosine	0.085
Tryptophane	0.028
Cystine	0.021

PROCESS OF IMMUNIZATION

Average m.l.d. of this toxin was found to be 0.00005 c.c. for 350 grammes guinea-pigs. To test the antigenicity of this toxin, 15 horses were taken for trial. These horses had been regularly yielding antitoxic serum of fairly constant titre with toxin prepared with Witte peptone broth. Average titre of these horses were 1,800 international units per c.c. and this titre remained as before after re-immunization with the toxin produced in the medium herein described. Eleven fresh horses were then tried with this toxin and, after the usual full course of toxin, average titre obtained was 1,430 international units per c.c.

From these observations, it was considered to be of interest to introduce this medium in routine immunization. Up till now more than 200 horses have been satisfactorily immunized with this toxin. No serious allergic symptoms have yet been noticed in any of them.

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AN IMPROVED METHOD OF GROWING PURE CULTURES OF RINGWORM FUNGI

BY

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AND

P A MAPLESTONE, D S O, D S C, M B, B S D T M

(*Medical Mycology Inquiry, Indian Research Fund Association,
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[Received for publication, June 3, 1941]

It is difficult, particularly in the tropics, to obtain pure cultures of ringworm fungi from the skin, because the skin is always heavily contaminated with pyogenic cocci and other bacteria which on account of their relatively rapid growth on ordinary culture media prevent the slower-growing fungi from becoming established

We have tried various methods of attaining this end, such as —

- (1) Inoculating the primary culture on to tubes of media that have been allowed to become quite dry
 - (2) Drying the scales in an incubator and cutting them into very small pieces before placing them in the culture tubes
 - (3) Washing the scales for five minutes in alcohol and then twice in ether for two or three minutes before placing them on the culture media
- None of these methods has proved satisfactory, but it should be noted that method (2) has been found very good in the case of hair ringworms. We have also tried special media, such as that of Czapeck designed with this special object in view, but we have found that they inhibit the fungi as well as the bacteria

The recent work of Maplestone and Dey (1941) on the fungistatic and bactericidal properties of a group of substances, including some dyes, suggested another method, namely, mixing a dye of proved efficacy with the culture media

As a preliminary test we mixed one drop of 1 in 1,000 solution of gentian violet with 10 c c of Sabouraud's maltose-agar, while it was still in the fluid state. This was fairly successful and we got pure cultures of fungi more readily than we had ever done before. We then used two drops of gentian-violet solution in the same amount of medium and although the bactericidal effect was improved the appearance of the fungus was delayed too long, and sometimes altogether inhibited.

With these results as a basis we proceeded to work out the optimum strength of gentian violet that would inhibit the growth of bacteria and still permit the growth of fungus. Maplestone and Dey (*loc cit*) found that gentian violet inhibits the growth of a strain of *Staphylococcus aureus* in a dilution between 1 in 76,000 and 1 in 90,000 and that its fungistatic power did not extend beyond 1 in 5,000, so there was apparently a wide margin to work with.

Conditions of the experiments—Throughout the series gentian violet (Merck B) dissolved in distilled water in a strength of 1 in 1,000 was used, and the strength of dye in the medium was varied by adding different quantities of this solution to a fixed amount of the culture medium. The same culture medium was used throughout, it was Sabouraud's standard medium, except that glucose was substituted for maltose because the latter was not obtainable on account of the war. In making the dye impregnated media the required amount of standard gentian-violet solution was mixed with 100 c c of medium while it was still liquid, it was then poured into test-tubes and sloped in the usual way. The medium was sterilized before the gentian violet was added, to obviate the chance of chemical changes being induced in the dye by heat. This necessitated the final stages in the culture tube preparation being carried out under strictly aseptic conditions.

Infected scales of skin or nails were washed in alcohol for about five minutes, they were then cut in very small pieces and again washed in alcohol, and finally they were placed on the slopes, care being taken that no two fragments were in contact.

After a series of experiments involving observations on 202 separate cultures in which amounts of standard gentian-violet solution varying from 1 c c to 0.20 c c to each 100 c c of medium were used it was found that 0.25 c c gave the best results. It will be noticed that this is an apparent strength of only 1 in 400,000 gentian violet, much higher dilution than that found effective by Maplestone and Dey. We noticed that after a day or two the particles of skin or nail in the cultures had taken on a distinct violet tinge which probably means that the dye diffuses from the culture medium into the particles of dead tissue so that in the latter its concentration becomes greater than it is in the culture medium, which offers a reasonable explanation of the great difference between the effective dilution used in the manner employed by Maplestone and Dey (*loc cit*) in their experiments and by ourselves in the present instance.

SUMMARY

A simple method of impregnating culture media with a dye is described. It is effective in preventing growth of bacteria, while it allows free growth of ringworm fungi of the skin.

REFERENCE

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Note—The species of fungus used in the experiments was *Epidermophyton floccosum*.

PLATE XIII



Fig 1



Fig 2



Fig 3

Fig 1—Control tube containing plain Sabouraud's medium, and showing heavy contaminating growth of bacteria and no growth of fungus

Fig 2—Tube with 0.5 c.c. of gentian violet solution to 100 c.c. of Sabouraud's medium and showing no growth of bacteria or fungus

Fig 3—Tube with 0.25 c.c. of gentian violet solution in 100 c.c. of Sabouraud's medium with no contaminating growth and a single large colony of *Epidermophyton floccosum*

Duration of culture—one week

CALCIUM INTAKE AND FLUORINE POISONING IN RATS

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THE subject of fluorine poisoning has attracted considerable attention within recent years and extensive reviews of the subject are available (McClure, 1933, Roholm, 1937, Greenwood, 1940). One of the signs of fluorine poisoning is 'mottled enamel' which tends to occur when drinking water contains 1 to 2 parts per million of fluorine or more. This dental condition has been reported in certain districts of the Madras Presidency (Shortt, McRobert, Barnard and Mannadi Nayar, 1937, Raghavachari and Venkataramanan, 1940), and also in the Punjab by Wilson (1939) and Day (1940).

Fluorine poisoning may cause lesions of the bones in human beings. Roholm (*loc cit*) has observed chalky white bones with irregular surfaces and widespread periosteal deposits and calcification of ligaments, in cryolite workers. Apparently similar lesions have been observed in adults in the Nellore district of the Madras Presidency (Shortt, Pandit and Raghavachari, 1937). In the neighbouring Kurnool district, a disease of the bones among cattle, which may be of similar origin, is common and causes much economic loss. The problem of fluorine poisoning is thus of considerable importance in India.

Suggestions have been made that the toxic effect of fluorine is influenced by the composition of the diet in various factors. Pronounced C avitaminosis has been thought to be a contributory factor in the production of severe chronic fluorine intoxication in man (Pandit, Raghavachari, Subba Rao and Krishnamurthi, 1940). A diet rich in calcium, phosphorus and vitamin D is also stated to have a beneficial influence on fluorine intoxication (Roholm, *loc cit*, Smith, Lantz and Smith, 1935). McClure and Mitchell (1931) found a negative calcium balance with large intakes of fluorine, and it has been suggested that in such circumstances the latter monopolizes the calcium of the organism (Roholm, *loc cit*). If this were so, the severity of fluorine intoxication should be inversely proportional to the calcium content of the diet. The relation between calcium intake and the effect of fluorine poisoning in rats has been studied in the present investigations.

EXPERIMENTAL

Young, growing albino rats of about 50 g to 60 g body-weight were used, in groups of six animals each (three males and three females) Four groups were fed on the following diets —

- GROUP I A basal diet of starch 60 parts, casein 20, gingelly oil 8, cod liver oil 2, dried yeast 5 and salt mixture 5 The composition of the salt mixture was as follows calcium lactate 39 parts, calcium phosphate 16.2, iron citrate 3.54, potassium phosphate 28.62, sodium phosphate 10.41, magnesium sulphate 7.98, sodium chloride 5.19 and potassium iodide 0.5
- „ II The same diet as group I plus 0.05 per cent of sodium fluoride Each rat consumed approximately 4 mg to 6 mg of sodium fluoride per day
- „ III The same diet as group II but containing the salt mixture minus its calcium salts (calcium lactate and calcium phosphate)
- „ IV The same diet as group III but without the sodium fluoride This group served to control the effect of the low intake of calcium in group III

The experiments were continued for 336 days, when the surviving animals were killed Most of the animals survived, except for those in group III, in which all animals died within the first 12 days Another batch of six young rats of the same age and weight was selected and fed on the group III diet In this repeated experiment, the average survival period was only 9 days

The animals in group II showed typical symptoms of chronic fluorine poisoning, the teeth presenting a chalky appearance and growing inwards, the latter were brittle and 'mottled' in a few instances Bone lesions resembling those described by Roholm (*loc cit*) in rats also occurred These were not observed in the animals dying within a short period The animals grew for the first few weeks after which they remained more or less stationary in weight The survival period of the animals in the various groups and the average weekly increase in body-weight are shown in Table I —

TABLE I

Survival of rats as influenced by sodium fluoride and calcium content of the diet

Group	Average survival period, days	AVERAGE WEEKLY CHANGE IN BODY WEIGHT IN G DURING THE PERIOD OF SURVIVAL	
		At the end of 5 months	At the end of the experiment
I	336	3.77	2.81
II	207	3.00	2.54
III	11	-8.50*	
„ Repeat	9	-9.50*	
IV	336	3.50	2.75

* At death which took place within 2 weeks

It is apparent from Table I that fluorine administered in the doses mentioned above was highly toxic when the dietary intake of calcium was low or insufficient for the body needs of the animals. Less than 0.1 per cent of calcium was present in the diet of group III. The same dose of fluorine did not rapidly kill the animals in group II which received much larger amounts of calcium. The calcium content of the diet of group II was about 0.7 per cent.

To throw further light on the problem, another series of experiments was carried out. Some groups received the same amount of fluorine as in the first series (0.05 per cent sodium fluoride) with varying intakes of calcium. In other groups the calcium intake was kept constant at a low level (0.1 per cent) and the dose of sodium fluoride varied. Experiments were also undertaken to discover whether calcium present in natural foodstuffs has a beneficial effect in 'de-toxicating' fluorine. Ragi (*Eleusine coracana*) and polished raw rice, the former being rich in calcium and the latter poor, were substituted for starch in the synthetic experimental diet and two groups of rats were fed on these modified diets respectively. The effect of a reduced phosphorus intake was also studied. Except in group XI the surviving animals were killed after 280 days.

The following diets were fed to groups of six young rats --

- Group V The same diet as group II but with 0.5 of the amount of calcium salts in the salt mixture (the diet contained 0.35 per cent of Ca)
 VI The same diet as group II, but with 0.25 of the amount of calcium salts in the salt mixture (the diet contained 0.18 per cent of Ca)
 VII The same diet as group IV plus 0.025 per cent of sodium fluoride
 VIII The same diet as group IV plus 0.01 per cent of sodium fluoride
 IX The same diet as group III with ragi substituted for starch
 X The same diet as group III with milled raw rice substituted for starch.
 XI The basal diet plus phosphorus free salt mixture plus 0.05 per cent of sodium fluoride

The survival period and growth of the animals are set out in Table II --

TABLE II
Effect of varying calcium and fluorine intake

Group	Average survival period days	Average weekly change in body weight in g during the period the animal survived
V	102	2.18
VI	23	-0.70
VII	32	1.40
VIII	280	3.13
IX	55	1.64
X	21	1.11
XI*	238	2.60

* The experiment with this group was started later, the surviving animals were killed after 238 days.

Table II shows that the addition of increasing doses of calcium to experimental diets containing a constant percentage of fluorine correspondingly increased the survival periods of the animals. Likewise, progressively decreasing doses of fluorine given with a diet low in calcium proportionately increased the survival rate. Thus, with 0.01 per cent sodium fluoride in an almost calcium-free diet, the animals survived for 280 days with fairly satisfactory growth, putting on over 3 grammes weight per week on an average during a period of 40 weeks, whereas when the dose of sodium fluoride was increased to 0.025 per cent, the diet otherwise remaining the same, the animals exhibited poor growth and died within 5 weeks. A similar result was obtained when natural foodstuffs, one (rice) poor in calcium and the other (ragi) rich in it, were substituted for starch in the synthetic diet. The animals on the basal diet containing ragi (*Eleusine coracana*) survived for 55 days, while those on the basal diet containing rice survived for only 21 days. The mitigating influence of calcium on fluorine poisoning is, therefore, obvious. A change in the phosphorus intake (group XI) appeared to be without influence.

It should be observed that the doses of sodium fluoride employed in these experiments were above the human level of intake in areas of endemic fluorosis. Most of the animals receiving sodium fluoride and surviving for long periods showed the characteristic bony lesions.

Fluorine in relatively large doses is stated to cause a negative calcium balance (Roholm, *loc cit*), presumably because of the formation of calcium fluoride, a relatively insoluble salt. For the formation of this salt, the stoichiometric relation between calcium and fluorine is approximately 1:0.95. In the experiments in which calcium was observed to produce a mitigating effect on fluorine poisoning, over 30 times as much calcium was given as that necessary for combination with fluorine to form calcium fluoride, and smaller amounts of calcium did not produce the same protective effect. It does not therefore seem likely that the action of calcium can be wholly explained as being due to the removal of fluorine as an insoluble salt.

SUMMARY AND CONCLUSION

Calcium, administered either as a salt or as present in combination in natural foodstuffs, exerted a mitigating influence on fluorine poisoning in rats. At the levels of dosage used, the toxic effect of fluorine was inversely proportional to calcium intake.

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697

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THE VITAMIN D CONTENT OF SOME FISH OILS.

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As a result of the war, imports of cod-liver oil into India have practically ceased. Considerable attention has been given to the production of cod-liver oil substitutes and at present these are being extensively used. Cod-liver oil owes its importance in medicine to its content of vitamins A and D. While a good deal of research has been carried out on the vitamin A content of Indian fish-liver oils, e.g. shark-liver oil, used for the manufacture of cod-liver oil substitutes, little work has been done in India on their vitamin D content, the only paper on the subject being that of Basu and Sen Gupta (1940), who record the vitamin D content of the liver and body oils of 10 Bengal fish. Vitamin D is of importance in the treatment of rachitic diseases which are common in North India. Hence it is desirable to have information about the vitamin D content of fish oils available in India. The present paper records the results of the vitamin D assay of 14 fish oils.

EXPERIMENTAL

Vitamin D was assayed by the 'line test' technique based on the effect of test substances in promoting calcification in the bones of rats fed on a rachitogenic diet. Young rats from a healthy colony, weighing about 40 g to 45 g, were used. No special precautions were taken to keep the vitamin D content of the mother's diet low in vitamin D, but litters of 5 and more were removed at birth along with the mothers to a dark room, where they were kept during the period of weaning and throughout the experiment. The experimental animals were fed on a modification of Steenbock's rachitogenic diet No. 2965 (Steenbock and Black, 1925), yellow maize and wheat gluten being replaced by similar amount of whole-wheat flour and egg white. The composition of the diet was as follows —

	Parts
Whole-wheat flour (atta)	76
Egg, white	20
Calcium carbonate	3
Sodium chloride	1

Plus one drop of a vitamin A concentrate (2,000 international units) every 14 days

The above diet had a calcium-phosphorus ratio of approximately 4 : 1 and was found to be satisfactory as a rickets-producing diet

About 30 young rats were put on the experimental diet simultaneously. During a preparatory period of 3 weeks the basal diet only was given. The animals were then divided into four groups, care being taken to distribute litter mates throughout the groups. One group, consisting of 3 to 6 animals, was kept on the basal diet and served as a negative control, another group, including 8 rats, was given 25 international units of vitamin D in the form of the international standard preparation as a single dose, the third and fourth groups, of about 8 animals each, were given the test oils in varying doses. The vitamin D content of the latter was estimated from the amount required to produce a degree of 'healing' or calcification comparable to that produced by the dose of the international standard.

After 10 days the animals were killed and the distal ends of the radius and ulna removed. The bones were split longitudinally, and one half, with the cut surface uppermost, exposed to sunlight in silver nitrate (1.5 per cent), for 2 minutes, and subsequently for the same period in distilled water. The blackened 'line' caused by the formation of colloidal silver at the epiphysis serves as an index of 'healing' and was assigned a value according to standards given by Coward (1938) for the line test. The assessment of 'healing' values was made by at least two independent workers without prior knowledge of the group to which the animals belonged. The average amount of 'healing' induced in each group was then evaluated, and by a comparison of these values with those given by the standard vitamin D under the experimental conditions, the vitamin D content of the test samples was determined.

Oils studied — The object of the investigation was to discover how much vitamin D is present in shark and saw-fish liver oil, now being used in the manufacture of cod-liver oil substitutes, and to find out whether any fish oil available in large quantities was a rich source. An oil with a potency of the order of that of tuna fish oil, which contains about 40,000 international units per gramme, would be of great value for admixture with other oils. Liver oils from fish caught in only small quantities, however rich in vitamin D, would not be of much use in the manufacture of medicinal preparations on a large scale and were not included in the investigation. The Table sets out the vitamin D content of 14 samples of fish oil, as determined by the 'line' test —

TABLE
The vitamin D content of some fish oils

Fish oils	International units per gramme
1 'Finest Norwegian' cod liver oil	180
2 Shark-liver oil	150

TABLE—concl'd

Fish oils	International units per gramme
3 Shark liver oil	210
4 do	220
5 do	220
6 do	260
7 Diluted shark liver oil* the diluent was probably ground nut oil	Very low
8 do do	Very low
9 Saw fish liver oil (<i>Pristis</i> group)	200
10 do do	180
11 Sardine oil	200
12 Mackerel oil	220
13 Ray oil	160
14 Plough fish oil (<i>Rhincobatus diadema</i>)	250

* Containing 1,000 international units of vitamin A per g

It will be seen from the Table that the fish oils examined were not excessively rich in vitamin D. The shark-liver oils had an average vitamin D content of 210 international units per gramme, a value about twice that of B P cod-liver oil. The Norwegian cod-liver oil tested gave a value higher than the B P specification. Bills (1935) records a lower content of vitamin D for one sample of shark-liver oil.

DISCUSSION

The vitamins A and D potency of B P cod-liver oil is 1,000 and 100 international units per gramme respectively. Shark and saw-fish liver oils are, on an average, 8 to 10 times richer than this in vitamin A, and only 1.5 to twice as rich in vitamin D. When the manufacture of a medicinal preparation from these oils was undertaken in India it was decided to aim at a vitamin A potency of about 1,500 international units per gramme, i.e. 50 per cent above the B P standard. Preparations standardized at this value could be given in the same dosage as cod-liver oil, to which the medical profession was accustomed. In order to reach this standard, ground-nut oil was used as a diluent. Another advantage of dilution lay in the fact that batches of shark-liver oil vary in vitamin A potency and a uniform product can be produced by the suitable admixture of a vegetable oil.

Such a diluted product will contain less vitamin D than cod-liver oil, which experience has shown to be suitable as regards concentration for anti-rachitic medication. If the dose is adjusted to supply sufficient vitamin A, too little vitamin D may be given. Hence, if shark and saw-fish liver oil preparations, diluted with vegetable oils, are to be used in the prevention and treatment of rachitic conditions, it is desirable that they should be fortified with a vitamin D concentrate, preferably calciferol. One gramme of calciferol would 'vitaminize' about 100 gallons of blended oil as described above, giving a vitamin D potency of about 100 international units per gramme in the final product.

SUMMARY AND CONCLUSION

Fourteen samples of fish oil were tested for vitamin D by the 'line' test. The value of undiluted oils ranged from 150 to 260 international units, with an average of about 200 international units per gramme. The need for 'fortifying' cod-liver oil substitutes based on shark and saw-fish liver oil is discussed.

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INFANTILE MORTALITY IN THE BERIBERI AREA OF THE MADRAS PRESIDENCY.

BY

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THE epidemiology of beriberi in India was discussed in a previous communication from the Laboratories (Aykroyd, Krishnan, Passmore and Sundararajan, 1940), in which it was shown that beriberi as a serious public health problem is confined to the Northern Circars district of the Madras Presidency. Its limited geographical range was explained on the grounds that in the Northern Circars the majority of the population consumes raw rice, whereas in other rice-eating areas in Madras, and throughout the rice-eating parts of India generally, the poorer classes prefer parboiled rice. Raw rice loses most of its vitamin B₁ when machine milled to a high degree, whereas parboiled rice retains as much as two-thirds of its anti-beriberi vitamin when treated in the same way, this being due to the diffusion of the vitamin through the endosperm in the steaming process. About 70 per cent of the rice-eating population of the Madras Presidency consumes machine-milled rice, and this practice is as common outside the beriberi area as within it.

In all Eastern countries in which beriberi is prevalent, the disease is known to be common among infants. According to reported figures, about one-third of the deaths from beriberi in Japan during the years 1928-32 were in infants under one year, while in Manila in the Philippines during the same period the proportion of infantile deaths was about one-half (Burnet and Aykroyd, 1935). Beriberi in infants has not hitherto been reported in India, but experience in other countries strongly suggests that it *must* be common in the beriberi area. A brief preliminary note on this question has already been published (Aykroyd and Krishnan, 1941).

During visits to the Northern Circars we have had the opportunity of observing clinically cases of infantile beriberi. Our attention was first drawn to the condition by Sister Mary of the Sacred Heart, Medical Officer in St Joseph's Hospital, Guntur. Sister Mary found that breast-fed infants, seriously ill or even at the point of death from causes previously obscure, could be dramatically relieved by the injection of pure vitamin B₁. Subsequent inquiries in hospitals and out-patient departments have convinced us that infantile beriberi is common in the beriberi area, though as yet usually unrecognized.

Clinical picture—The following brief description is based on textbook accounts and on our own observations —

Beriberi usually occurs in breast-fed infants aged 3-4 months. Often the child fails to gain in weight during a week or so previous to the attack. In the acute form the infant is suddenly seized with what appear to be severe paroxysms of pain, during a paroxysm it may straighten out its body and become quite rigid. Between attacks, the muscles feel abnormally soft and flabby. Vomiting is frequent. There is usually cyanosis and coldness of the extremities and difficulty in breathing is obvious. Right-sided dilatation of the heart is common and the pulse is weak and rapid. Excretion of urine is diminished. General cedema is rare but localized cedema may be observed. A husky or almost inaudible voice, due to cedema of the larynx and not to paralysis of the vocal cords, is very characteristic of infantile beriberi. The knee-jerks may be absent. Death often occurs within 24 hours unless the appropriate treatment is given.

In more chronic cases the usual clinical picture is that of a pale flabby baby, not gaining in weight and becoming weaker and weaker. Vomiting and constipation are frequent. Infants with chronic beriberi may die suddenly of heart failure.

Examination of the mother will often reveal evidence of beriberi in the form of slight paresis, difficulty in walking and numbness and tingling in the extremities. She will often describe the sudden death of previous infants at the age of 3-4 months or thereabouts.

The therapeutic test is the most satisfactory method of diagnosis. Injection of pure synthetic vitamin B₁ may relieve an infant which appears to be almost moribund. In very acute cases less concentrated preparations, such as yeast or extract of rice polishings, may not have time to act, but they may be effective when the condition is less grave—witness the success achieved in the Philippines in the treatment of infantile beriberi with 'tiki-tiki', an alcoholic extract of rice polishings.

Trend of infantile mortality in the Northern Circars—Epidemiological investigations in hospitals and out-patient departments present considerable difficulties. In this preliminary inquiry we have approached the problem by a study of the statistics of infantile mortality in a number of towns in the beriberi area and in other places. The Annual Reports of the Director of Public Health, Madras (1935-39), provide data about infantile mortality in municipalities in the Madras Presidency. Infantile deaths are grouped under the periods 0-1 month, 1-6

months, and 6 months to 1 year. A comparison was drawn between the proportionate mortality in these periods in 17 towns in the Northern Circars and 17 towns in the province south of Madras City in 1938, the total population concerned being approximately the same in each case. Health officers were employed in nearly all the towns during the period under consideration and this means that registration of births and deaths is likely to be fairly accurate.

In the beriberi area a greater proportion of infant mortality occurred in the period 1-6 months than in the periods 0-1 month and 6-12 months. In the other towns and in British India generally, the greatest proportionate mortality occurred during the first month. The reported infantile mortality in the towns in the beriberi area was considerably higher than in the towns outside the area (*vide* Table).

TABLE
Infantile mortality in 34 towns (1935-39)

	Reported average infant mortality rate	0-1 month Per cent of total infant mortality	1-6 months Per cent of total infant mortality	6-12 months Per cent of total infant mortality
17 towns in beriberi area	203	34.2	41.8	24.0
17 towns outside beriberi area	164	52.7	26.0	20.4

All observers agree that beriberi usually attacks infants at the age of 3-4 months. A study was made of the available records of infantile mortality in the age group 0-6 months, at each month of life, comprising the years 1935-39, in the following six municipalities —

Beriberi area

Bezwada

Cocanada

Vizagapatam

Other towns

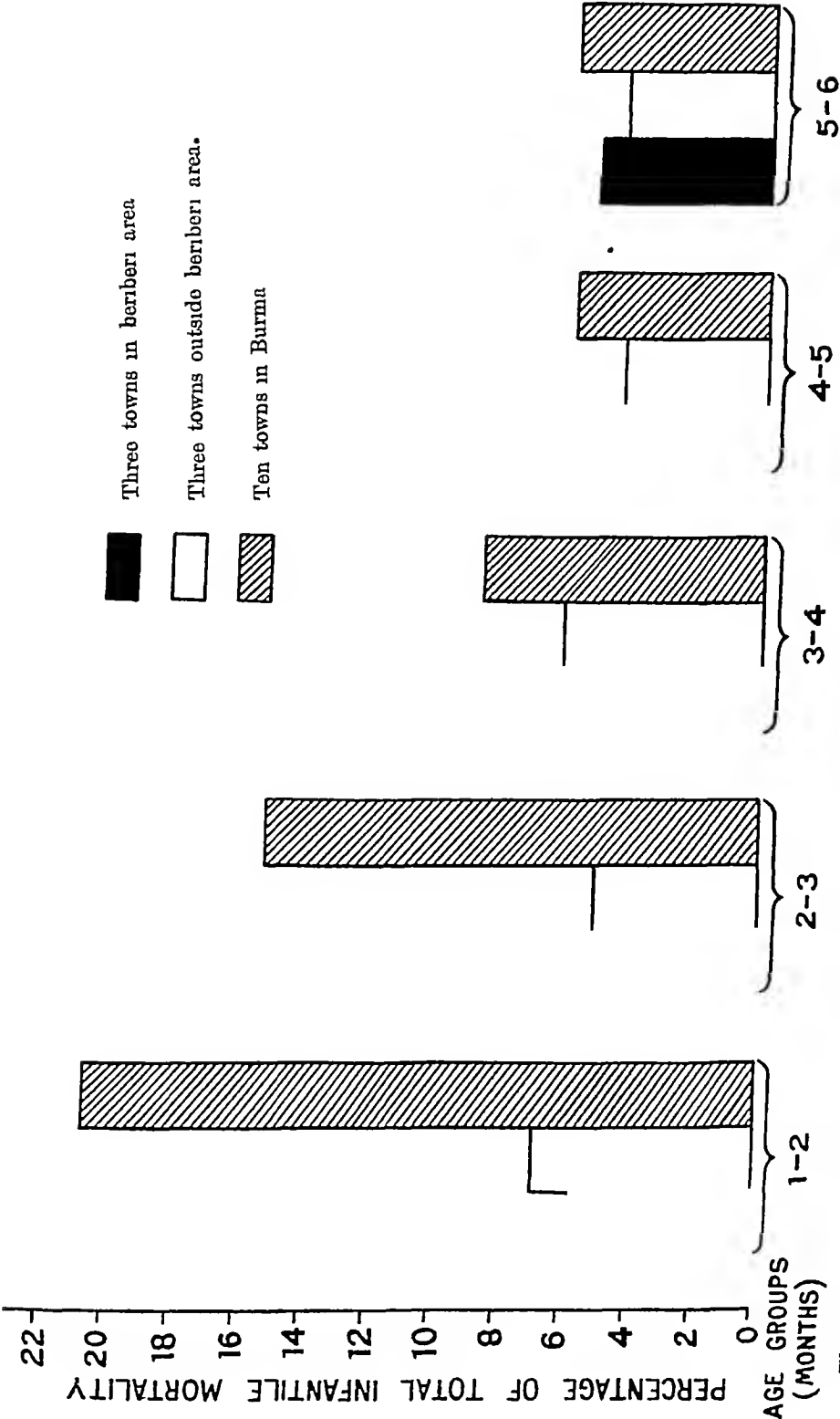
Kumbakonam.

Tanjore

Tuticorin.

The results are shown in the Chart. In the towns in the beriberi area, there was a sharp and constant peak at the fourth month of life, which was not visible in the records of the other towns. Causes of death—debility, diarrhoea, convulsions, etc.—were included in the municipal records, but these mean very little and no account was taken of them. The inaccuracy of vital statistics in India is well known. But a mother whose child dies in infancy usually knows approximately its time of birth and we can think of no reason why more deaths should be consistently recorded as occurring in the fourth than in other months except the obvious one that more deaths actually occurred in the fourth month. The peak in mortality at this period of life revealed itself only on detailed examination of the records, its existence was unknown to the municipal health officers who had collected them. It may be pointed out that registration of births and deaths in

CHART.



The percentage of total infantile mortality occurring at each month in the age period 1-6 months, in three towns in the Northern Circars, three towns in another district of the Madras Presidency, and ten towns in Burma (1935-39)

the Madras Presidency is more satisfactory than in most parts of India and that we were concerned with urban areas in which whole-time health officers were employed

In the Annual Report of the Public Health Commissioner with the Government of India (1936) it is pointed out that in Burma the proportionate mortality at different periods of the first year of life is very different from that recorded in British India. The figures given are as follows —

Deaths.	Under 1 month	1-6 months	6-12 months.
British India (1936)	47.3	31.3	21.5
Burma (1936)	23.5	58.0	18.5

Data relating to monthly infant mortality in a number of towns in Burma were kindly supplied by the Director of Public Health and the Nutrition Officer, Burma. The percentage monthly mortality for ten towns for 1935-39 is shown in the Chart. It will be seen that while infant mortality in Burma remains high during the first 6 months of life, there is no *peak* at the fourth month. Beriberi does not appear to be common in Burma, although precise information of its incidence is lacking. The Public Health Commissioner's Report (*loc cit*) suggests that the high mortality in Burmese infants aged 1-6 months is due to the practice of feeding infants with solid food after the first month of life and probably this explanation is the correct one.

An effort was made to obtain monthly records of infantile mortality from an area in which beriberi is known to be common, namely Singapore. Unfortunately the subdivision of age periods in Singapore does not correspond with the Indian classification, being 0-3 months and 3-12 months. Records kindly sent by Dr. Faris, Chief Health Officer, Singapore, show that in this area infant mortality after the neonatal period (0-1 month) is greater than in India, but no further comparisons can usefully be drawn at this stage. It is the practice among the Malays to feed infants in solid foods in the early months of life and this, as in Burma, may influence the trend of infantile mortality.

DISCUSSION

Clearly the above observations, though suggestive, do not *prove* that beriberi is an important cause of infantile mortality in the Northern Circars. Proof can only be obtained by field investigations in which the cause of infant deaths is carefully investigated and trial is made of the effect of vitamin B₁ on sick infants. It is hoped to carry out investigations of this nature in the near future. Meanwhile the attention of health officers and medical practitioners in the beriberi area may be drawn to the possibility that illness and death of obscure causation occurring in breast-fed infants at about the fourth month of life may be due to vitamin B₁ deficiency.

SUMMARY.

1 Cases of infantile beriberi have been observed in the Northern Circars district of the Madras Presidency, where adult beriberi is common

2 In three towns in this area a peak in infant mortality occurs at the fourth month of life This peak is absent in towns in which adult beriberi is rare or absent

ACKNOWLEDGMENTS

We are grateful to Sister Mary of the Sacred Heart, St Joseph's Hospital, Guntur, for supplying much useful information and for showing us cases of infantile beriberi, and to the Health Officers of the six municipalities in the Madras Presidency for co-operating in the study of their records Thanks are due to Major T J Davidson, I M S', Director of Public Health, Burma, Dr U Maung Gale, B A , M B , D P H , Nutrition Survey Officer, Burma, and Dr D W G Faris, Chief Health Officer, Singapore, for sending data about infantile mortality

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DENTAL CARIES IN INDIAN CHILDREN

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AN enormous amount of clinical, epidemiological and experimental work has been carried out in the investigation of the problem of dental caries. With regard to its ætiology, however, confusion and uncertainty still reign. Kungelmaas, Newton and Bodecker (1934) remark that the study of groups immune or relatively immune to the disease might throw more light on causation than the study of populations showing a high incidence. There has been a general impression that dental decay is much less common in India than in Northern Europe and America. In the course of numerous diet surveys rough-and-ready dental inspections, without the use of probe and mirror, have been made and have in general supported the idea that the condition of children's teeth in India, as far as caries is concerned, is relatively good. Taylor and Day (1939) observed a low incidence in children in the Kangra valley, Northern Punjab, and Day and Tandon (1940) have carried out an investigation in another group of children in the Punjab and recorded that the amount of caries present was less than in American children. Apart from these investigations, no other studies appear to have been made.

The present paper records the dental examination of 6,866 children in various parts of India.

METHOD OF INVESTIGATION

A detailed dental inspection, in which all the available tooth surfaces in the mouth were examined, using a dental mirror and probe, was made in the case of each child. The total number of teeth, and the number of deciduous and permanent teeth, were recorded. All extracted teeth were recorded as carious except the negligible percentage known to have been lost as a result of trauma or extracted for

some other reason In recording the extent of caries the classification of Day and Sedwick (1934) was employed —

- 1 Initial caries including softened or discoloured pits and fissures giving lodgment to a fine explorer
- 2 Freely accessible approximate cavities and small open cavities involving less than one-fourth of the tooth
- 3 More extensive caries involving more than one-fourth and less than two-thirds of the crown
- 4 Caries involving from two-thirds to complete destruction of the crown

The author is a medical graduate and has no qualification in dentistry In order to fit himself to undertake the investigation he underwent a course of training, lasting a few weeks, under Dr Marshall Day at the de Montmorency College of Dentistry and the Punjab Dental Hospital, Lahore Dr Day, the Principal of these institutions, very kindly demonstrated methods of detecting and assessing caries in its various stages It is necessary to mention the limitations of the author's experience in dental work because it is possible that he may have omitted to record the presence in some cases of very fine carious pits or fissures which a highly experienced dentist might include under 'initial caries' As regards the more pronounced degrees of caries, he feels that his records are satisfactory and accurate Exactly the same standards of assessment were applied in the case of all children examined so that the records of the incidence in different groups are strictly comparable

In addition to the dental findings, the following information was also recorded —

1 Age Date of birth was taken from school registers The accuracy of the registers, however, cannot be guaranteed, because many families, especially in rural areas, do not keep any record of the date of their children's birth

2 Sex

3 Residence rural or urban area

4 Diet nature of staple cereal

5 Economic status In urban areas, where the income of the families could be ascertained, the children were grouped as follows —

Group	I	Up to Rs 25 per month
"	II	From Rs 25 to Rs 50 per month
"	III	From Rs 50 to Rs 75 per month
"	IV	Rs 75 and over per month

The results were recorded on a suitable card

The following groups were studied —

Group	District.	Number of children
I	Delhi Province, rural	991
II	„ „ urban	1,074
III	Central India (Ajmer), urban	178
IV	Madras Presidency Kurnool district rural	2,822
V	„ „ Coimbatore, urban	969
VI	Nilgiris urban	605
VII	„ Nilgiris, Anglo Indian Orphanage	227

RESULTS OF INVESTIGATION

The percentage incidence of caries in all children is given in Table I, which shows also the degrees of caries and the average caries figure for each tooth. The 'total caries figure' is calculated by multiplying the numbers included under each degree of caries by 1, 2, 3, or 4 as the case may be. The 'average caries figure' is $\frac{\text{total caries figure}}{\text{number of teeth examined}}$.

This method has been followed by other investigations. It expresses the extent of caries as a factor which can be used in making comparisons between groups.

Table II shows the incidence in boys and girls. Table III compares caries in urban and rural children in Delhi Province. The urban children were all resident in Delhi City, while the rural children lived in villages some 15 miles from the city. These children were all wheat-eaters. In the last column the percentage of caries in all urban and rural children is shown, except that the Anglo-Indian children in the Nilgiris were not included.

In Table IV the incidence in wheat- and rice-eating children is compared. The groups included are I, II, III, V and VI. Table V shows incidence in relation to economic status in groups II, III and V. Only urban children are included. Table VI gives figures for caries in Indian day school children in the Nilgiris and for Anglo-Indian children in an orphanage in the same district.

TABLE I

(a)

Percentage of dental caries (6,866 children)

Age	Number of children	Percentage free from caries	DECIDUOUS TEETH		PERMANENT TEETH		All teeth percentage carious
			Number	Percentage carious	Number	Percentage carious	
5	70	58.5	1,416	6.0	29		6.1
6	294	55.4	5,796	7.6	1,161	2.5	6.8
7	458	44.3	7,175	9.7	3,438	3.5	7.8
8	648	39.0	8,608	11.7	6,557	3.6	8.2
9	494	39.2	5,469	13.8	6,619	3.0	7.9
10	640	44.3	4,952	12.6	10,619	2.9	6.0
11	462	46.1	2,201	12.7	9,697	3.5	5.2
12	813	42.8	2,301	8.3	18,219	3.9	4.3
13	631	47.2	387	11.3	16,796	4.0	4.1
14	622	47.7	119	16.8	17,238	4.3	4.4
15	527	44.7	31		14,744	4.4	4.4
16	489	41.3	13		13,794	5.1	5.1
17	709	45.9	2		20,788	4.5	4.5

(b)

	Total number of teeth	Percentage of teeth carious	EXTENT OF CARIES				Average caries figure
			1	2	3	4	
Deciduous	38,670	10.8	1,413	1,454	746	584	0.23
Permanent	139,679	4.0	4,025	999	393	264	0.06

TABLE II
Sex incidence of dental caries

Sex.	Age												
	5	6	7	8	9	10	11	12	13	14	15	16	17
	Percentage of teeth carious.												
Females	7.2	7.4	9.5	9.4	8.8	6.6	4.4	3.7	3.7	4.1	4.7	6.9	5.0
Males	5.0	6.0	6.5	7.6	7.3	5.7	5.6	4.6	4.2	4.4	4.4	5.1	4.5

		Total number of teeth	Percentage of teeth carious	EXTENT OF CARIES				Average caries figure
				1	2	3	4	
Deciduous	F	14 253	11.6	544	573	322	226	0.25
	M	24 417	10.8	869	881	424	358	0.21
Permanent	F	26 822	3.9	727	180	82	53	0.06
	M	112 857	4.1	3,298	819	311	211	0.06

TABLE III
Dental caries in rural and urban children, Delhi Province

Age	Rural or urban	Number of children.	Percentage free from caries	DECIDUOUS TEETH		PERMANENT TEETH		ALL TEETH PERCENTAGE CARIOUS	
				Number	Percentage carious	Number	Percentage carious	Delhi.	All
5	R	2	100.0	40	0.0				
	U	12	58.3	260	14.1				
6	R	54	68.5	1,024	3.5	254	3.1	3.5	4.1
	U	69	49.2	1,643	11.9	388	1.4	9.8	9.2
7	R	128	57.0	2,007	6.6	1,055	3.0	5.4	4.4
	U	170	36.0	2,739	13.2	1,330	3.0	9.9	9.8
8	R	145	53.1	1,930	6.9	1,612	2.9	5.1	5.9
	U	151	26.4	1,994	16.2	1,646	4.0	10.7	10.3

TABLE V
Economic status and the incidence of dental caries (urban children, Delhi and Coimbatore)

Group	Family income	Age											
		6	7	8	9	10	11	12	13	14	15	16	17
		Percentage of teeth carious											
I	Up to Rs 25 per mensem	7.7	5.0	8.4	8.6	6.8	7.4	4.2	4.1	4.3	3.8	5.4	4.3
II	" Rs 50 "	7.5	12.8	9.7	8.8	6.6	6.5	5.5	3.3	4.4	3.9	5.3	5.1
III	" Rs 75 "	8.6	7.2	10.8	10.0	6.3	6.6	6.5	5.7	4.2	4.6	3.9	4.6
IV	More than Rs 75 "	10.9	11.4	12.5	10.7	7.0	6.2	4.1	4.3	3.9	4.3	5.3	4.7

TABLE VI

Dental caries in Anglo-Indian and Indian children (Nilgiris)

Age	Anglo-Indian and Indian children	Number of children	Percentage free from caries	DECIDUOUS TEETH		PERMANENT TEETH		All teeth percentage carious.
				Number	Percentage carious.	Number	Percentage carious.	
7 {	A. I	2		29	24.1	19		12.5
	L	15	46.0	232	9.0	94		6.3
8 {	A. I	8	37.5	114	6.1	76	7.8	6.8
	L	22	40.9	333	12.6	192	1.5	8.5
9 {	A. I	8	12.5	70	28.5	122	8.1	15.6
	L	15	28.6	170	20.5	194	2.5	10.9
10 {	A. I	13	15.4	91	25.2	187	4.2	11.2
	L	25	28.0	179	20.6	429	5.2	10.1
11 {	A. I	13	30.7	26	23.0	296	7.7	9.0
	L	44	31.8	192	27.6	936	7.3	10.8
12 {	A. I	31	25.8	105	12.3	744	6.7	7.4
	L	85	43.5	207	11.1	2,046	6.1	6.5
13 {	A. I	29	10.3	10	70.0	780	7.3	10.6
	L	80	46.2	45	11.0	2,174	4.8	4.8
14 {	A. I	33	30.3	8		910	9.1	9.0
	L	84	54.7	21		2,388	4.1	4.2
15 {	A. I	35	11.4			976	10.6	10.5
	L	59	49.1			1,647	4.2	4.8
16 {	A. I	25	12.0			706	11.8	11.8
	L	90	43.3			2,549	5.9	5.9
17 {	A. I	29	6.9			750	11.6	11.6
	L	71	53.5			2,075	5.1	5.1

		Total number of teeth.	Percentage of teeth carious.	EXTENT OF CARIES				Average caries figure
				1	2	3	4	
Deciduous	A. I	479	17.3	25	31	16	11	0.37
	L	1,413	11.1	70	91	58	39	0.41
Permanent	A. I	5,586	9.5	271	141	62	58	0.17
	L	14,763	5.1	490	184	59	46	0.08

DISCUSSION OF RESULTS

The percentage of all children free from caries was 44.5 (Table I). King (1940) gives the following figures for the percentage of children free from caries in London, Sheffield, and Lewis (an island off the north-west coast of Scotland). These may be compared with the Indian figure —

London	1.9
Sheffield	2.5
Lewis (rural)	28.1
Lewis (urban)	2.0
India	44.5

The age groups concerned are approximately comparable in each case. The incidence of caries in Indian children was much lower than that recorded in children in the United States (Day and Sedwick, 1935). The results of the present investigation thus support earlier impressions about dental caries in India. More caries was present in deciduous than in permanent teeth, which explains the higher incidence in children aged 6 to 9. As regards deciduous teeth, the extent of caries was somewhat greater in girls than in boys (Table II). The proportion of the sexes in the various groups, including the Anglo-Indian school, was approximately the same, so that the slightly higher incidence of caries in deciduous teeth in girls does not invalidate comparison of the general incidence in all children in the different groups.

The incidence of caries was consistently higher in urban than in rural children in all age groups (Table III). A much more pronounced difference, to the advantage of rural children, was noted by King (*loc cit*) in Lewis. A considerable proportion of children in Delhi City are, however, children who have spent some of the earlier years of life in rural areas. Some children come from surrounding country areas to attend schools in the city. Many of them were children of workers in the Government of India Secretariat, who come from different parts of the country. Some of these families may have been resident in Delhi for only a few years. A higher incidence of caries in urban than in rural children was observed in rice-eating as well as in wheat-eating areas. Here again a fair percentage of children of rural origin were included in the urban group.

Wheat-eating children (Table IV) had less caries than rice-eating children. The difference was evident in all the age groups except 7 and 8, in which the number of rice-eaters was small.

An attempt was made to grade the children in Delhi and Coimbatore schools into different economic groups, by obtaining information from the school authorities, and in some instances from the child itself, about the pay and occupation of parents. The data obtained were probably not very accurate in some cases. No consistent difference in the incidence in children included in the various groups was observed (Table V). It is to be observed that the majority of children belonged to families of low economic status.

The Anglo-Indian children in the Nilgiris showed a higher incidence of caries of permanent teeth than any other group (Table VI). The average caries figures

for permanent and deciduous teeth in these children, Indian day school children in the Nilgiris, and all Indian children, were as follows —

	Deciduous	Permanent	All teeth
Anglo Indian children, Nilgiris	0 37	0 17	0 19
Indian children, Nilgiris	0 41	0 08	0 10
All Indian children	0 23	0 06	0 09

The average caries figure in the Anglo-Indian children was thus about double the general average for all teeth, while for permanent teeth, it was about three times the general average and double the figure for Indian children in the Nilgiris, who showed the highest incidence among Indian children. The Nilgiris Indian children had actually more caries in the deciduous teeth than the Anglo-Indian children.

The percentage of children free from caries in the orphanage was only 17.6, as compared with 44.5 for all the Indian children and 45.7 for Indian children in Coonoor. The number of pronounced cavities in the teeth of the boarding school children was greater than in all other groups. Of permanent teeth, 2.2 per cent showed degrees 3 and 4 as compared with 0.7 per cent in Indian children in the same district, and 0.4 in the whole Indian group.

For the sake of brevity, the incidence of caries in the Kurnool district, in which the staple cereals are millet and rice, is not recorded separately. It corresponded approximately to that obtaining in the wheat-eating children. Dental fluorosis was very common in this area and it is proposed to consider the relation between caries and 'mottled enamel' in a later communication.

Few bleeding gums were seen, and spongy gums were rare. The presence or absence of gross hypoplasia was not recorded, but it can be definitely stated that this condition was uncommon. In general the teeth surfaces in Indian children are smooth and regular.

Diet—The majority of the rural wheat-eating children included in the investigation were resident in the Najafgarh district, Delhi Province, in which diet surveys have already been carried out (Shourie, 1939). While the most important cereal in this area is whole wheat, consumed in the form of unleavened cakes or 'chapattis', millet (*Pennisetum typhordeum*) is also consumed to a considerable extent. Intake of milk products is fairly high, the following daily intake per consumption unit having been recorded in a group of cultivators (Shourie, *loc cit*) —

	Oz
Milk and butter-milk	14 0
Ghee	0 5

The diet contained fairly large quantities of pulses (3 oz to 5 oz daily) but intake of vegetables was low and that of fruit negligible. Little meat was

included in the diet. No diet surveys have been made in Delhi City. Here also the diet is based on whole wheat and contains milk in fairly abundant amounts.

In the rice-eating groups the diet consumed was of the type often described in previous communications from the Laboratories. In a typical poor rice diet, rice supplies 80 to 90 per cent of total calories. Intake of pulses is usually in the neighbourhood of 1 oz., of non-leafy vegetables, 2 oz. to 6 oz., of vegetable oil, less than 1 oz. Leafy vegetables are taken in small quantities while the consumption of milk is usually negligible. Consumption of meat, fish and eggs does not often exceed 1.5 oz., and as a rule not even this amount is eaten. Fruit is a rare ingredient in the diet. In the millet-eating area the diet resembled the poor rice-eater's diet in its content of non-cereal foods, the main difference being the replacement of rice by millet (*Sorghum vulgare*). Precise data about intake of refined or unrefined sugar cannot be given. It can, however, be assumed that the vast majority of children included in the survey consumed sugar in amounts far below those consumed by the average European and American children.

Certain conclusions with regard to diet and dental caries may be drawn from the investigation. The consumption of rice diets poor in most of the important vitamins including vitamin D, and in calcium, and generally very deficient by European standards, is compatible with *relative* freedom from dental caries. Again the diet of the Indian child, whatever the staple cereal, contains few 'hard' foods which require vigorous chewing. Rice diets are particularly soft and pappy. The investigation thus gives no support to the familiar theory that dental decay is caused by the excessive consumption of soft 'over-cooked' foods.

Oral hygiene was conspicuous by its absence in the Indian children. Poor children in India do not clean their teeth. The fact that there is no relation between oral hygiene and caries is now generally recognized.

The possible rôle of vitamin D deficiency in causing dental decay has been the subject of much discussion and investigation during recent years. The generally low incidence of dental caries in India is compatible with the vitamin D deficiency hypothesis, since in countries bordering on, or in, the tropics more vitamin D is supplied by the action of sunlight on the skin than in the temperate zones. On the other hand, less caries was observed in Delhi in latitude 28° than in Madras at latitude 12°. It is well known that rickets and osteomalacia are more common in North than in South India. Taylor and Day (*loc cit*) observed a low incidence of caries in children showing clinical evidence of rickets in the Northern Punjab. Little evidence of gross rickets was, however, observed in the course of the present investigation among children in Delhi City or Delhi Province.

The relatively higher incidence of caries in the Anglo-Indian orphanage is a matter of considerable interest. Most of the children in the orphanage had been resident there since early childhood. Judged by the usual standards, the diet in this institution was a good one, in certain respects superior to that of the wheat-eating Indian children and in nearly every respect superior to that of the rice-eating

groups The expenditure on food was Rs 8 to Rs 9 per child per month, which allows a varied diet to be supplied The chief cereal food of the children, at the time of the dental examinations, was bread made from refined wheat flour, the daily ration of which was 12 oz Under-milled wheat in small quantities was consumed as a breakfast porridge and small amounts of rice and dhal were also supplied The allowance of milk products was the liberal one of one pint of whole milk and $\frac{1}{2}$ oz of butter per child daily The daily diet also included about 4 oz of potatoes and about 12 oz of other vegetables and fruits The vegetable ration included a fair proportion of green leafy vegetables Meat intake was about 2 oz, possibly a little less Consumption of sugar was not less than 2 oz per child daily, which included molasses given at supper time to spread on the bread The children also had the opportunity of buying sweets in the school shop once a week up to the amount of their weekly pocket money of 4 annas The amount of sugar so obtained cannot be estimated, probably in the case of certain children fond of sweets, it was not inconsiderable

The above diet would be regarded as satisfactory in any children's charitable institution in England Some allowance must be made for kitchen leakages, but the home is a well-managed one and these were probably cut down to a minimum The general health of the children was good and their growth rate satisfactory They were accustomed to spend plenty of time in the open air, with bare arms and legs, and there is no reason to suppose that the amount of vitamin D obtained by the action of the sun was less than in the case of the Indian children in Coonoor (Table VI) who showed a lower caries incidence in the permanent teeth Actually the Coonoor day school children, without organized games, drill, walks, etc would receive less direct sunlight than the children in the orphanage

No definite reason can be assigned for the greater amount of caries among the Anglo-Indian children, and the same may be said of the relatively higher incidence in Indian children in the Nilgiris, as compared with other Indian children As regards the Anglo-Indian children, however, attention may be drawn to the high intake of sugar, which was much higher than in the other groups, and to the considerable quantities of white bread consumed We know that in countries in which the incidence of dental caries is high, these two foods bulk largely in the diet

It is clear that at the present time dental caries is not a problem of major public health importance in India Nearly half the children showed no caries at all, and severe decay calling for dental treatment was rare—at any rate in comparison with the state of affairs in Northern Europe and America Since in general the standards of health and nutrition of children in Indian schools are inferior to those of European and American children, it is of interest to note that they have the advantage at this particular point

SUMMARY

1 The extent of dental caries in 6,866 children aged 6 to 18 in various parts of India has been studied

2 The percentage free from caries was recorded as 44.5, a much higher percentage than that observed in England and U S A

3 The incidence of caries in deciduous teeth was higher in girls than in boys

4 Urban children in all age groups showed more caries than rural children. Wheat-eaters had a slight advantage over rice-eaters. No correlation between income and the extent of caries was observed.

5 The highest incidence in permanent teeth was observed in an Anglo-Indian orphanage, where the diet was in many respects superior to that consumed by the Indian children investigated, but included bread made from refined wheat flour and fairly large quantities of sugar.

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STUDIES IN THE VITAL CAPACITY OF BOMBAY MEDICAL STUDENTS

Part I

STATISTICAL CORRELATION WITH PHYSICAL MEASUREMENTS *

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THE vital capacity of the lungs represents the maximum range of the inspiratory and expiratory mechanisms of an individual and is gauged by the volume of air forced out by him by a maximum expiratory effort after a maximum inspiratory effort

The scientific study of vital capacity may be said to have commenced with Hutchinson (1846, quoted by Myers, 1925) who devised his special instrument for the purpose—the spirometer. In his extensive work in this field he observed the wide variations of this measure in apparently healthy individuals and he came to the conclusion that this could only be due to the variations in the physical builds of persons. He correlated the vital capacity with the various physical measurements and established the 'norms' of vital capacity for persons of different heights, etc. The deviations from these in the apparently healthy he associated with their relative unfit condition and poor physique and in the unhealthy with their diseases, especially those of the respiratory system. He, however, said that chest measurements bore no relationship to vital capacity.

The work that followed Hutchinson's broadly confirmed most of his findings. Attempts were made to establish the normal standards of vital capacity both for males and females especially on the Continent of Europe by Simon (1848),

* Paper read before the Physiology Section of the Indian Science Congress 1941

Fabius (1853), Schneevogl (1854) and Arnold (1855) (all quoted by Myers, *loc cit*) Interest was again renewed in this question by Bohr's (1907, quoted by Myers, *loc cit*) work in Copenhagen and by that carried out at Oxford in England by Schuster (1911) who studied it from the anthropometric aspect In America Peabody and Wentworth (1917) studied the question from the standpoint of disease

During the Great War of 1914-18 several tests were devised both in England (Dreyer, 1919, Flack, 1921) and in America (Schneider, 1920, quoted by Myers, *loc cit*) for assessing the physical fitness and efficiency of individuals for the purposes of recruitment to the various military services

The determination of the vital capacity was one of the tests used for recruitment to the Royal Air Force A good many of these tests were also done on the civil population, on men, women and children

Investigations on the vital capacity standards in other countries followed soon after, on the Chinese by Foster and Hseigh (1923) and McCloy (1927), on the Philippines by Myers (*loc cit*) and others, and on the Japanese by Satake and Sato (1938)

In India observations on vital capacity have been recorded by Bhatia (1929), Mason (1932), Krishnan and Vareed (1932, 1933) and De and De (1939)

Actual establishment of standards for normal individuals necessitated the working out of quantitative correlation between vital capacity and the various physical measurements including age Different statistical methods have been used by different workers for this purpose

Hutchinson (*loc cit*) himself pointed out a definite correlation between vital capacity and standing height, weight and age Fabius (*loc cit*) thought stem length or sitting height to be more intimately correlated with vital capacity than standing height Arnold (*loc cit*) stressed the relation between vital capacity and chest circumference as well as chest expansion and believed the constant difference existing in the results on males and females to be due to these factors

Schuster (*loc cit*) published correlation coefficients between vital capacity, standing height and weight Lundsgaard and van Slyke (1918, quoted by Jackson and Lees, 1929) attempted correlation between vital capacity and various trunk measurements Dreyer (1919) by careful observation on 16 subjects of ages between 13 and 52 evolved a set of power formulæ correlating vital capacity with each of the following measurements, viz standing height, stem or sitting height, weight, and chest circumference He also argued that stem (sitting) height was a better index of vital capacity than standing height and that power formulæ better fitted the observed data than the linear relations of Hutchinson West (1920) observed the best correlation between vital capacity and the body surface

area as calculated from Dubois's formula and gave ratios $\frac{\text{vital capacity}}{\text{standing height}}$ and $\frac{\text{vital capacity}}{\text{surface area}}$ which could be used as normal standards His conclusions, especially

those regarding surface area, were supported particularly by American investigators Cripps, Greenwood and Newbold (1923) working on Flack's (*loc cit*) data on Royal Air Force recruits observed that multiple linear regression equations

more accurately measured the relationship between vital capacity and the other measurements than Dreyer's power formulæ. The latter were also criticized by Mumford and Young (1923). Hewlett and Jackson (1922) calculated the correlation coefficients and the regression equations correlating vital capacity with height, weight and surface area. Jackson and Lees (*loc cit*) from observations on 100 selected university students also computed correlation coefficients and ratios between vital capacity and various measurements. McCloy (*loc cit*) working on the Chinese got the best correlation between vital capacity and surface area.

In India the only statistical work on the vital capacity of Indians that we could trace in the literature available was that by Mason (1932) on South Indian females. She has correlated vital capacity with standing height, weight and surface area and has devised linear regression equations between them separately. She has also published the calculated regression lines for these.

No work, however, of a statistical nature on males seems to have as yet been undertaken in India. With this object of investigating the correlation between vital capacity and other physical measurements by statistical methods and of constructing if possible suitable prediction formulæ we have collected, analysed and computed data based on the measurements of 172 apparently healthy male medical students of Bombay.

METHODS

Subjects—The subjects were all apparently fit and healthy male students in their first and second years of medical studies. All who gave a recent history of any illness, serious or trivial, were rejected. Those who gave a past history of any respiratory disorder such as pleurisy, asthma, hæmoptysis, etc., were also excluded. But for these the selection of cases was at random. Most of the students belonged to the Bombay Presidency, only a few hailing from outside. All religions were represented. Hence the racial or regional factor could not be considered in the analysis.

General—The observations were carried out between August 1939 and July 1940. The temperature of the room varied from 80.6°F to 87°F. Students were examined either between 11 a.m. and 12-30 p.m. or between 3 p.m. and 4-30 p.m. and at least three hours after any sort of meal or refreshment. Sleep during the preceding night and habits regarding diets, exercise and smoking were inquired into.

Measurements—The actual measurements were taken according to the following procedure—

Age—The age was recorded in complete years.

Standing height—This was taken without shoes on a special platform to which a vertical measuring rod, marked both in English and metric scale, was fixed. The height was recorded in centimetres to the nearest quarter of a cm.

Sitting height (stem length)—This was taken by seating the subject on a low stool, about 20 inches high, to which was fixed a measuring rod marked in the metric scale. The subject was made to flex his knees under the stool and to sit

erect against the scale The sitting height was also recorded in centimetres to the nearest quarter of a cm

Weight—This was taken with only the shirt and trousers on The machine used was a lever machine from Messrs Baird and Tatlock, Ltd, London The weight was observed in pounds to the nearest half pound and then converted into kilograms to the nearest quarter of a kg

Surface area (Dubois, 1924)—This was recorded in sq m by reference to the nomogram prepared by Boothby and Sandiford and based on Dubois's formula

Surface area (Dreyer, 1912-13, quoted by Dubois, *loc cit*)—This was calculated from Dreyer's formula, viz surface area = (weight)^{0.72} × 0.1231

Chest measurements—These were taken on bare chests at the level of the nipples with a tape marked in inches The subjects were made to stand with arms hanging by the side The measurements were in inches to the nearest quarter of an inch later converted into centimetres to the nearest quarter of a cm The *inflated chest circumference* was taken in the position of maximum inspiration and the *deflated chest circumference* in that of maximum expiration The mean between the two readings gave the *mean chest circumference*, while their difference measured the *chest expansion*

Trunk area—Two special measurements were calculated to represent approximately the real trunk area The first approximation was made by taking the product of sitting height × mean chest circumference and the second by taking the product of sitting height × inflated chest circumference, both calculated in sq cm

Pelldisi—von Pirquet's (1922, quoted by Mason, 1931) nutritional index was calculated from his formula, nutritional index = $\frac{\sqrt[3]{10 \times \text{weight (g)}}}{\text{sitting height (cm)}} \times 100$

Vital capacity—For this determination a wet type of chain compensated spirometer calibrated in c.c. as supplied by Messrs C F Palmer, Ltd, London, was used The subject was instructed previously to take the deepest possible inspiration and then to make a maximum possible effort to expel out the air into the spirometer through a glass mouthpiece connected to the rubber-tubing of the spirometer The subject was always standing while undergoing the test and the best of three efforts was recorded in each case

Statistical methods—The data obtained by the above methods were tabulated and the means, standard deviations and coefficients of variation with their probable errors worked out for each measurement Information regarding the distribution of the samples was thus obtainable We next computed by means of tables the correlation coefficients and the correlation ratios with their probable errors between vital capacity on the one hand and each of the other measurements on the other We could thus determine whether the association between vital capacity and the other measurements was significant or not and, if significant, whether the relationship was linear In those cases where a significant relationship was noticeable both calculated and observed regression lines were drawn and the regression equations

constructed. Finally from these the prediction lines for vital capacity for males were drawn against each of these measurements.

The methods followed in all these computations were those given by Pearl (1930). The formula for the probable error of the correlation ratio however which has not been given in Pearl's book was taken from Dunn's (1929) article on 'Statistical Methods in Physiology'.

RESULTS

The actual data obtained on 172 subjects are recorded in Table I.

The range of each measurement as also the nature of its distribution as given by the mean, the standard deviation, and the coefficient of variation with their probable errors are given in Table II.

The following points may be noted in these two tables.

The age range of our subjects is a small one—that of young adults between 18 and 29 years with a peak at 21 as shown in Graph 1.

On the whole the distribution of the measurements seems to be fairly normal as can be gauged from their means and standard deviations.

The coefficient of variation ranged from 3.63 for standing height to 19.08 for chest expansion. The least variable measurements were the standing and the sitting height, then came the chest measurements, while weight and vital capacity showed a comparatively greater variation. The coefficient of variation for vital capacity was 15.97.

Sitting height was equally variable as standing height and not more so as has been declared (Cripps *et al.*, *loc. cit.*)

Weight was more variable than surface area, while chest measurements were less so.

The mean vital capacity in the subjects studied was 2,950 c.c.

The surface area of the body as calculated by Dreyer's formula invariably gave higher figures than those obtained from Dubois's formula.

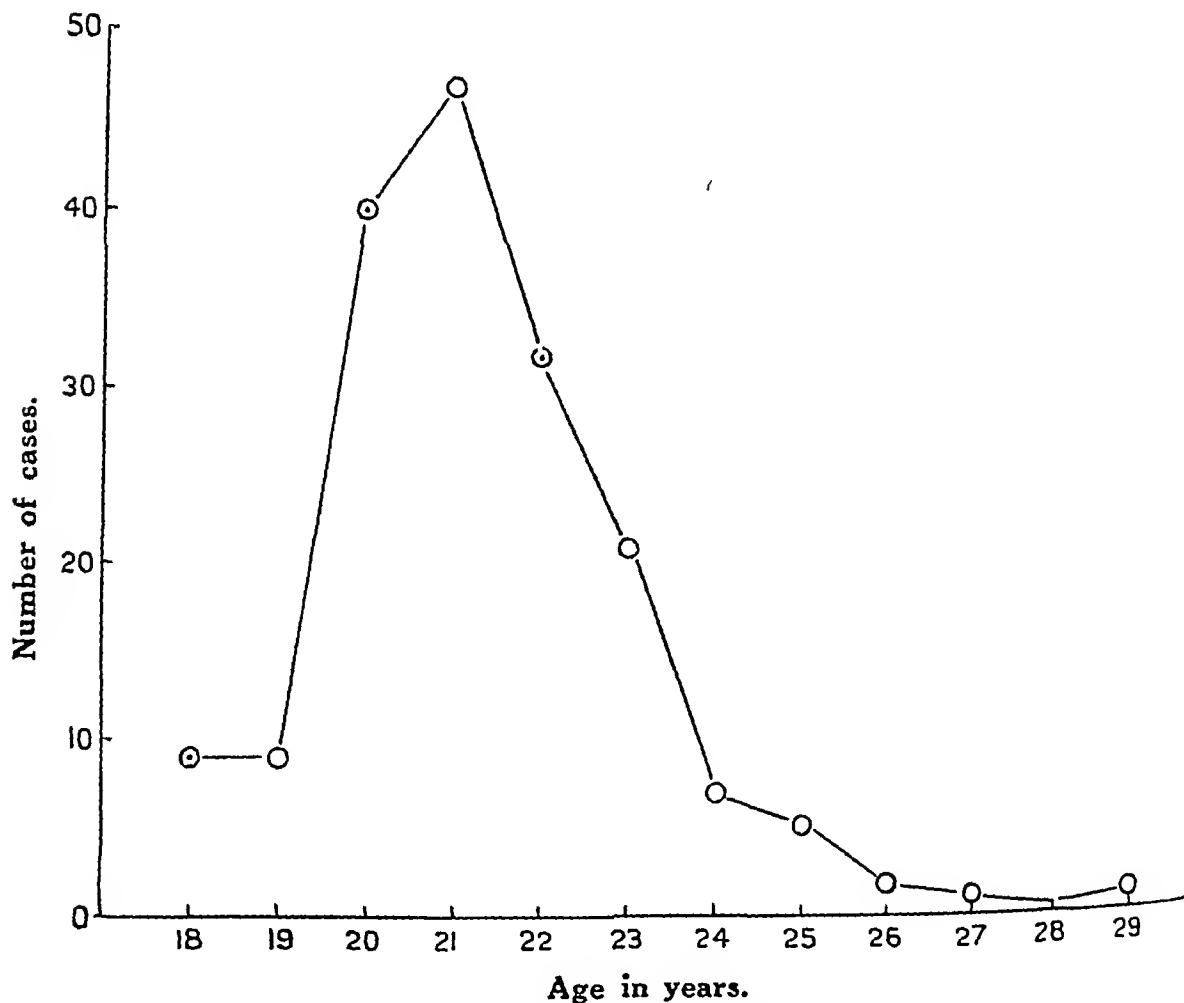
The correlation coefficients and correlation ratios of vital capacity against the other measurements are given in Table III. The measurements are tabulated in descending order of correlation obtained. The test for linearity as shown by $r^2 - ^2$ in each case is also included in the same table. The regression lines, observed and calculated, are shown in Graphs 2 to 14.

The following points in Table III and the regression lines may be stressed —

The relationship of vital capacity with age and Pelidisi is insignificant, but it is significantly correlated with all the other measurements to a greater or lesser degree.

The most significant correlation is with the trunk area calculated as the product of sitting height and inflated chest circumference, while closely on its heel follows

that with the sitting height. Next follow the correlation with standing height, trunk area (sitting height \times mean chest circumference), surface area (Dubois), inflated chest circumference, weight, and chest expansion in that order. The relationship with mean chest circumference, surface area (Dreyer), and deflated chest circumference which follow these is rather poor though not insignificant.

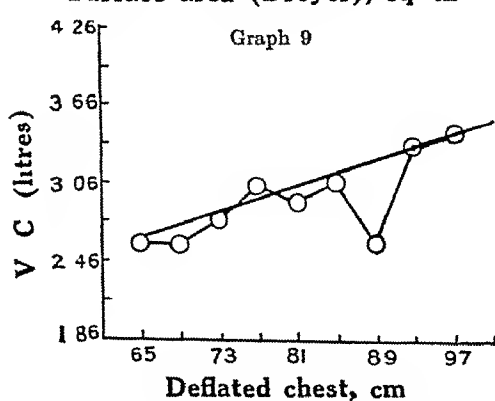
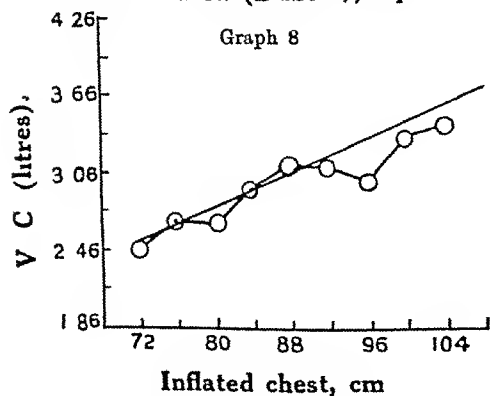
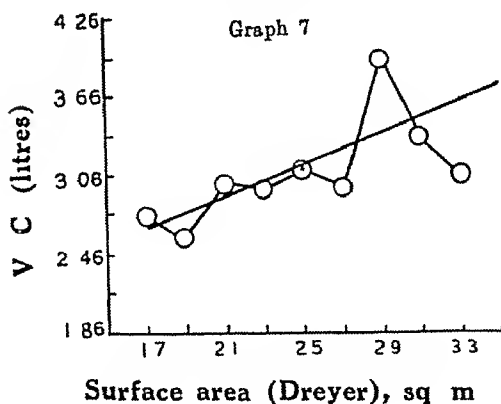
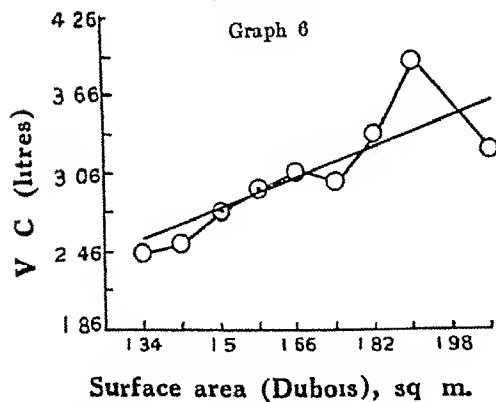
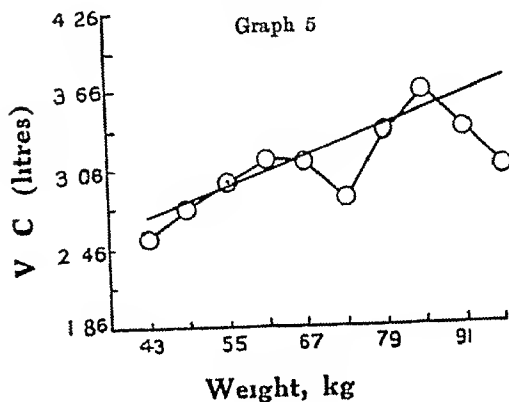
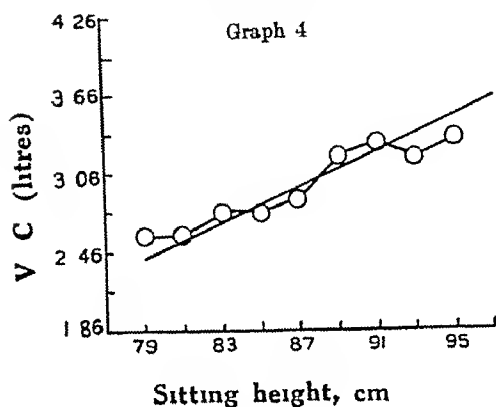
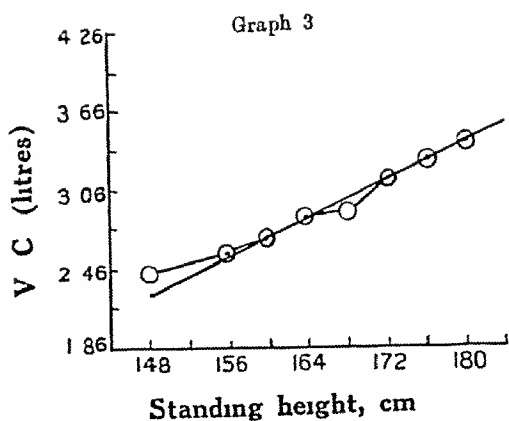
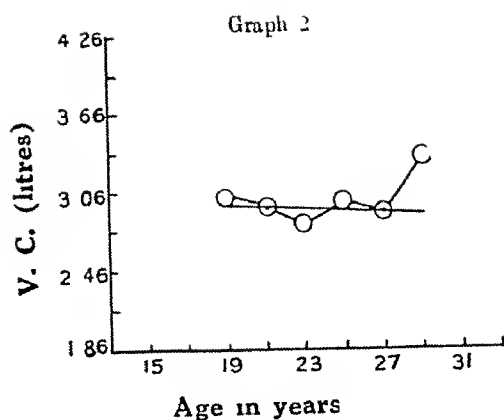


GRAPH 1—Age distribution of cases (total 172)

The figures for the correlation ratios follow closely those of the corresponding coefficients except in the case of age and Pehdasi.

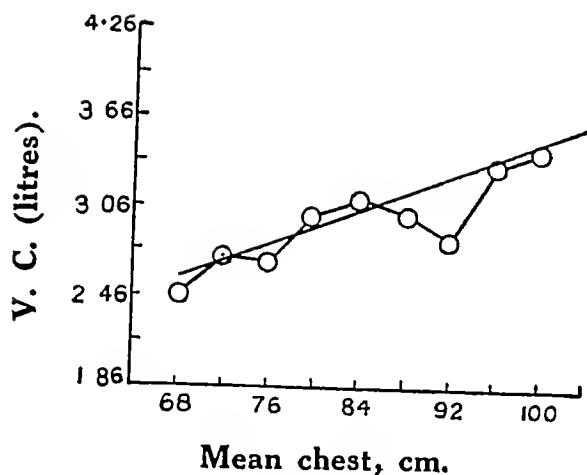
The linearity test applied according to Blakeman's (1905, quoted by Pearl, *loc cit*) criterion suggests that the relationship is linear in all the cases.

The regression equations of vital capacity in terms of the other measurements with which it is significantly correlated are given in Table IV and the prediction lines for vital capacity constructed from these equations in Graphs 15 to 25.

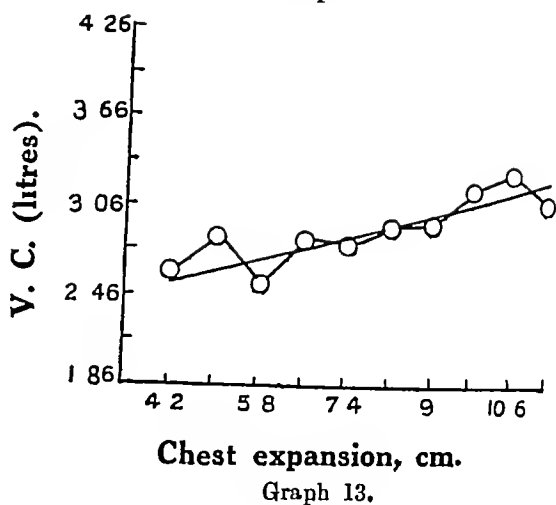


GRAPHS 2 to 9—Calculated (continuous line) and observed (interrupted line) regression lines of vital capacity on the various measurements

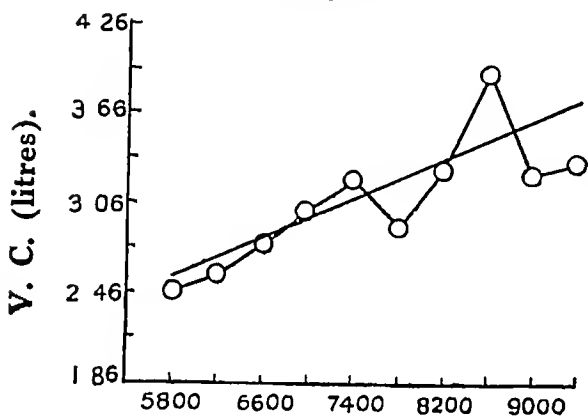
Graph 10.



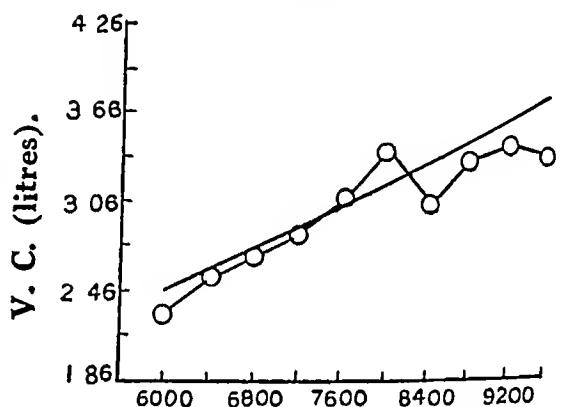
Graph 11



Graph 12



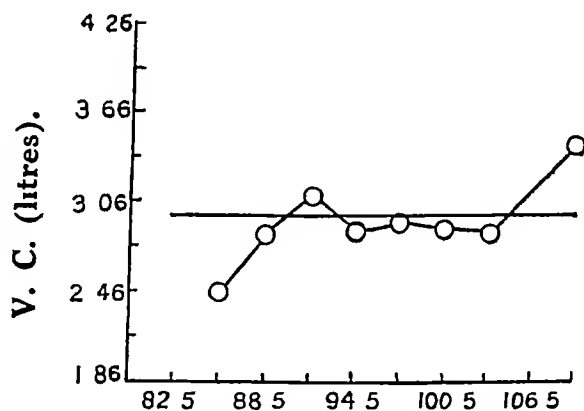
Graph 13.



Trunk area (sitting height \times mean chest), sq. cm.

Trunk area (sitting height \times inflated chest), sq. cm.

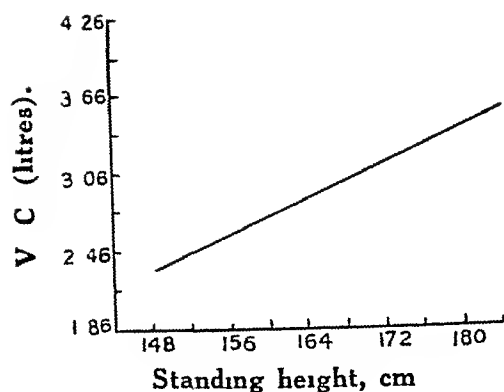
Graph 14



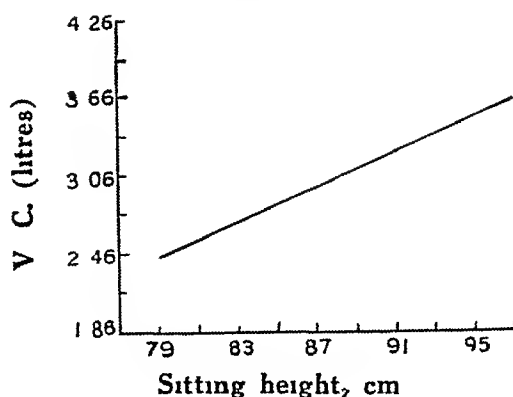
Pelidisi index.

GRAPHS 10 to 14 — Calculated (continuous line) and observed (interrupted line) regression lines of vital capacity on the various measurements,

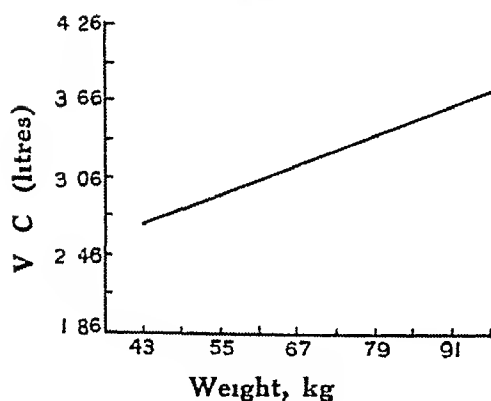
Graph 15



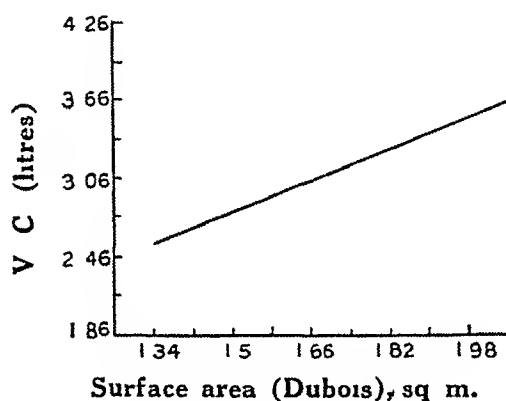
Graph 16



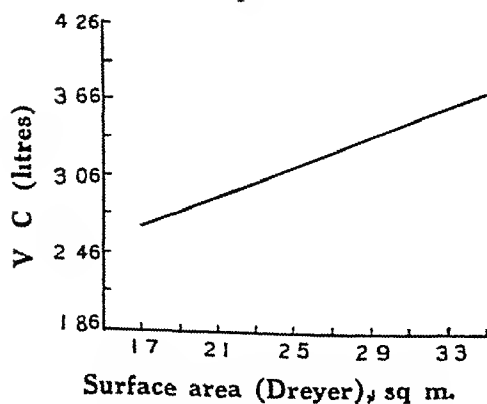
Graph 17:



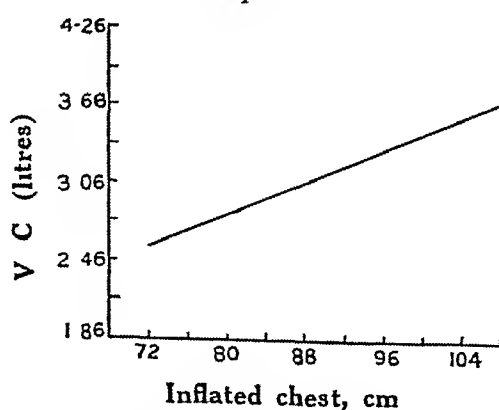
Graph 18



Graph 19

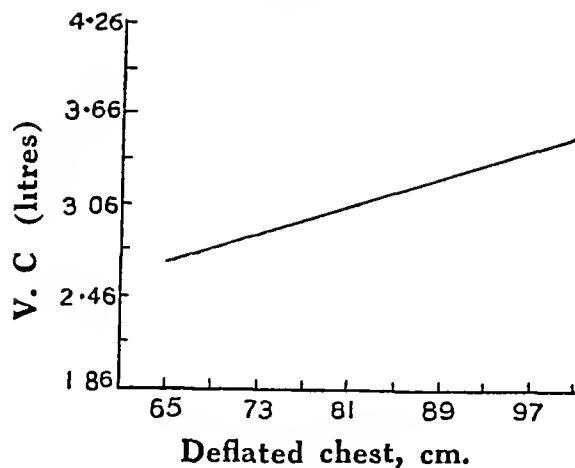


Graph 20

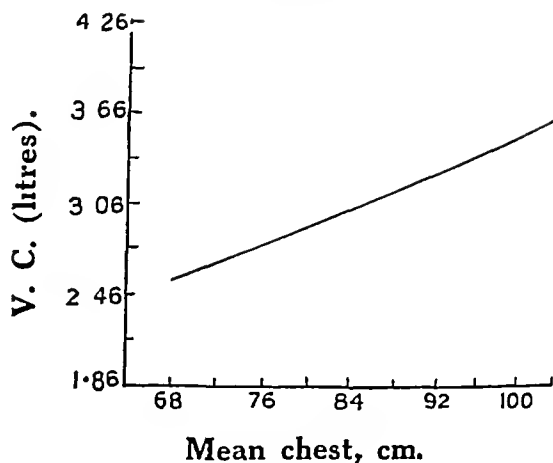


GRAPHS 15 to 20 —Prediction lines of vital capacity on the various measurements

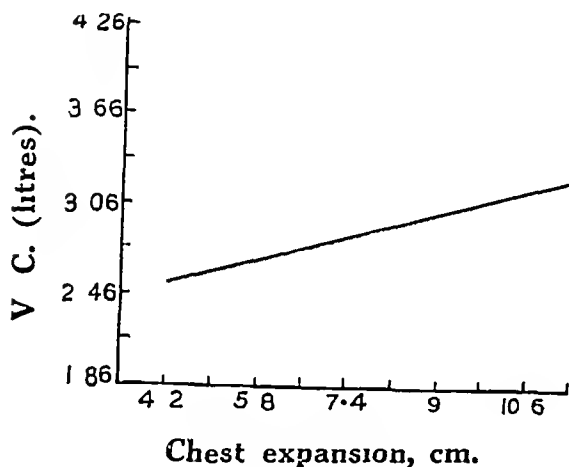
Graph 21



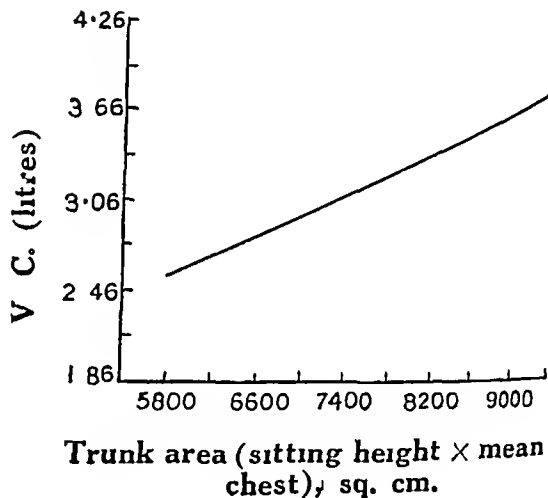
Graph 22



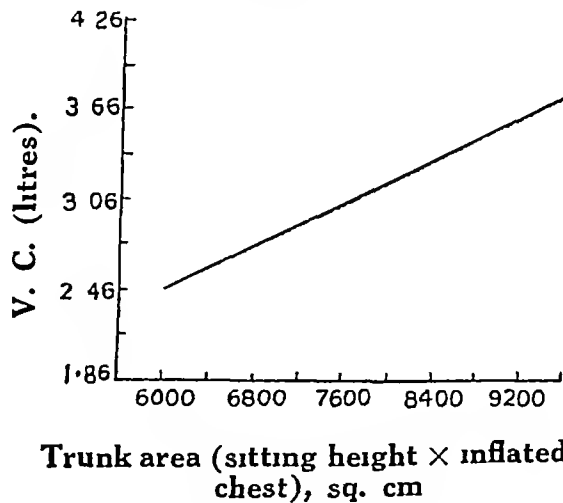
Graph 23



Graph 24



Graph 25



GRAPHS 21 to 25 — Prediction lines of vital capacity on the various measurements

CONCLUSIONS AND DISCUSSION

A comparative table of certain aspects of vital capacity figures obtained by Indian workers is given in Table V. A similar comparison showing the results of other investigators including observations on the Chinese, the Japanese, the English, and the American subjects is given in Table VI. Table VII shows a proportional comparison between the average Indian and the average non-Indian figures for vital capacity and Table VIII a comparison between the standing heights and body surface areas of Indians and non-Indians. The points that emerge from a study of all these and of the data presented by us in this paper follow

It will be seen that the results obtained on vital capacity by the different Indian workers at Bombay, Madras and Calcutta are nearly equal. The highest average figure of 3,096 c.c. was obtained by Bhatia in Bombay but the age range of his subjects was wider, viz. 20 to 45 years. Besides, possibly in his case, a dry type of spirometer may have been used. The age range of all the other subjects is practically the same.

The vital capacity figures for Calcutta as observed by De and De (*loc cit*) are rather lower than those of Madras or Bombay.

Compared with the vital capacity figures for the Chinese, the Japanese, the English or the American the Indian figures are all distinctly lower. The average Indian figure is 93 per cent of the Chinese, 78 per cent of the Japanese, 68 per cent of the average English and 65 per cent of the average American figure. The underlying cause of this lower figure may either be racial or due to other factors such as climate, diet, habits, etc. There is no doubt, however, that the disproportionately higher figures for the non-Indians to a great extent might be due to most of their observations being on selected groups of subjects rather than on subjects chosen at random as was done by us. Thus, Hutchinson's (*loc cit*) subjects were chosen at random and compared with his figures the Indian average is 82 per cent of the English average and not 68 per cent.

The vital capacity per cm. of standing height in our cases was 17.65 c.c. Amongst Indian cases it varied from 16.5 c.c. to 18.52 c.c., the average being 17.73 c.c. The nearest approach to this figure in the non-Indian subjects is that for the Chinese which is 19.5 c.c. The average Indian figure for this is 91 per cent of the Chinese, 76 per cent of the Japanese, 71 per cent of the English and 68 per cent of the American average. When random sampling is considered such as Hutchinson's the average Indian figure is 84 per cent of the English instead of 71 per cent.

Similarly, the vital capacity per sq. m. of body surface in our cases was 1,830 c.c., in the other Indian cases it ranged from 1,790 to 1,960 c.c., the average of all being 1,870 c.c. This figure again is 92 per cent of the Chinese, 78 per cent of the Japanese, 77 per cent of the English and 74 per cent of the American average figures. Compared with Hutchinson's figure based on random sampling the Indian figure is 93 per cent instead of 77 per cent of the English figure.

It is remarkable to note here that the Indian figures howsoever compared with the Chinese and the Japanese figures, that is to say, whether the average vital capacity or vital capacity per cm of height or vital capacity per sq m of body surface is compared, the Indian figure bears the same proportion to the Chinese or the Japanese figure, viz nearly 92 per cent of the Chinese and nearly 78 per cent of the Japanese figures in all the three comparisons. On the other hand, the proportion of the Indian to the English or the American figures varies according to the method of comparison. Thus, when the average vital capacity figures are compared the Indian figure is 68 per cent of the English and only 65 per cent of the American. As we compare the vital capacity per cm of standing height the Indian figure forms 71 per cent of the English and 68 per cent of the corresponding American figure and when the vital capacity per sq m of body surface is compared the Indian figure is 77 per cent of the English and 74 per cent of the American figure. This may be due to the fact that the comparatively high average vital capacity of the Chinese and the Japanese is possibly compensated by their general lower standing height and also by the consequent relatively larger body surface of these people when compared with that of the Indians (see Table VIII). It will be seen from Table VIII that while the average standing height of the Japanese and the Chinese is smaller than that of Indians their body surface is practically the same or a little higher than that of Indians, while the average height as well as the body surface of both the English and the American subjects are greater than those of Indians.

A better criterion for such comparison and of greater interest might have been the ratio of vital capacity to sitting height or of vital capacity to trunk area or trunk volume, but unfortunately such ratios have not been available in the literature, especially for the Chinese and the Japanese.

Another equally remarkable fact which may be observed from the comparative Tables V and VI is this that while in the case of the Indians, the Chinese and the Japanese, that is to say amongst the Oriental subjects in general, the ratio of vital capacity (c c) to body surface (sq m) is more than hundred times the ratio of vital capacity (c c) to standing height (cm) it is invariably less than hundred times the latter in the case of the English and the American subjects. This is most probably the result of the fact as will be seen from Table VIII that the standing height (cm) of the Indians, the Chinese and the Japanese is more than hundred times the body surface (sq m) figure while that of the English and the American is less than hundred times this figure. We do not know whether this has any biological significance. Incidentally the same table shows that the average height and the average body surface area of the Orientals are much less than those of the English and of the Americans.

Nothing can be said about the racial factor in vital capacity from the data presented.

On the statistical side no work has hitherto been done in India on the males. Mason (1932) has published some work on South Indians but her subjects were all females. It is therefore not possible for us to compare our results in this respect with those of other Indian investigators. Amongst non-Indians statistical work

on vital capacity has been published as referred to in the beginning of this paper. Compared with the figures of those workers our correlation coefficients are rather low. Most of these workers get the highest correlation with surface area and weight while we get it with height, both standing and sitting, and with the trunk areas. Our correlation figures with chest measurements also are not insignificant, while the general consensus of opinion is that the relation of vital capacity to chest measurements is not appreciable. Cripps, Greenwood and Newbold (*loc cit*) stress that the method of multiple regression is more reliable for prediction amongst multiple variables. We have had no time to work out the multiple regressions in our series and we hope to do so at some future day. As our statistical results do not tally with those given by non-Indian workers we have in this paper merely presented our results in this respect and we do not wish to draw any conclusions therefrom owing to the paucity of the data.

The prediction lines (Graphs 15 to 25) may prove useful in determining the normality of the observed vital capacity of adult males.

SUMMARY

1 Data are presented for the vital capacity and other physical measurements of 172 young, healthy, male medical students (age range 18 to 29 years) of Bombay.

2 Statistical distribution constants of these measurements and their statistical correlation with vital capacity are given. Most of the correlations are significant.

3 Regression equations of vital capacity in terms of the measurements which show a linear relationship with it (*viz* standing height, sitting height, weight, surface area, chest measurements, chest expansion and trunk areas) have been constructed and the prediction lines of vital capacity as calculated from these equations drawn.

4 The data for vital capacity are compared with those of other Indian and non-Indian workers and the results discussed in the light of the comparisons.

ACKNOWLEDGMENT

Our thanks are due to the subjects who volunteered for this work.

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Data of vital capacity and physical measurements

Serial number	Age in years	Standing height, cm.	Sitting height, cm.	Weight, kg	Surface area (Dubois) sq m	Surface area (Dreyer) sq m	Chest circumference inflated, cm.	Chest circumference deflated cm	Chest circumference mean cm	Chest expansion cm	Trunk area (sitting height \times mean chest) sq cm	Trunk area (sitting height \times inflated chest), sq cm	Pelvisi (von Parquet index)	Vital capacity, litres
1	20	172.75	80.75	55.0	1.64	2.20	86.3	76.7	82.5	7.0	7,190	7,180	94.4	2.7
2	21	171.5	88.5	97.0	2.08	3.31	102.8	98.5	99.7	6.3	8,820	9,100	102.0	2.02
3	23	160.25	83.75	48.5	1.48	2.01	80.0	73.0	76.5	7.0	6,410	6,700	93.0	2.4
4	21	163.25	83.75	55.0	1.57	2.20	85.1	77.0	81.5	7.2	6,820	7,120	97.8	2.42
5	22	173.0	91.75	62.0	1.73	2.30	82.5	75.5	79.0	7.0	7,250	7,570	93.0	2.58
6	22	172.0	80.0	52.25	1.6	2.12	83.8	73.0	78.4	10.8	6,080	7,400	90.5	3.08
7	19	178.0	86.75	59.5	1.73	2.32	86.3	80.0	83.2	6.3	7,220	7,480	97.0	3.06
8	23	172.25	80.0	51.75	1.60	2.10	86.1	76.2	80.7	8.0	7,180	7,570	90.2	3.34
9	21	161.50	83.0	57.5	1.60	2.26	81.5	78.0	81.3	6.5	6,750	7,010	100.0	2.52
10	20	160.25	88.5	47.5	1.53	1.97	78.7	72.4	75.6	6.3	6,000	6,000	88.2	2.4
11	21	169.75	91.0	52.5	1.59	2.14	86.3	76.2	81.3	10.1	7,400	7,850	88.7	3.02
12	22	170.5	88.5	57.5	1.66	2.26	86.3	76.5	81.4	9.8	7,200	7,640	94.0	3.02
13	23	168.0	87.0	57.25	1.62	2.25	83.8	76.2	80.0	7.6	6,000	7,200	95.4	2.56
14	22	165.5	81.0	46.75	1.40	1.97	82.5	72.4	77.5	10.1	6,280	6,680	95.8	3.06

TABLE I—*contd.*

Serial number	Age in years	Standing height, cm	Sitting height, cm	Weight, kg	Surface area (Dubois), sq m	Surface area (Dreyer), sq m	Chest circumference inflated, cm	Chest circumference deflated, cm	Chest circumference mean, cm	Chest expansion, cm	Trunk area (sitting) height \times mean chest, sq cm	Trunk area (sitting) height \times inflated chest, sq cm	Pelvis (von Parquet index)	Vital capacity, litres
15	21	161.75	84.0	45.75	1.45	1.93	76.2	67.9	72.1	8.3	6,060	6,400	91.7	2.64
16	20	166.5	84.0	48.5	1.52	2.01	76.8	69.2	73.0	7.6	6,130	6,450	93.5	2.04
17	20	161.5	85.0	60.5	1.63	2.36	87.1	80.0	83.6	7.1	7,100	7,400	99.5	2.94
18	23	177.5	92.0	87.25	2.07	3.07	99.1	92.7	95.9	6.4	8,820	9,120	104.0	3.38
19	23	172.5	90.0	50.0	1.65	2.24	86.3	78.7	82.5	7.6	7,430	7,770	91.6	2.72
20	20	163.5	82.75	51.0	1.53	2.09	83.8	76.2	80.0	7.6	6,620	6,930	96.6	3.12
21	21	167.75	85.5	63.0	1.70	2.43	88.9	80.0	84.5	8.9	7,230	7,600	100.0	2.42
22	20	170.25	84.75	51.0	1.58	2.09	81.9	74.3	78.1	7.6	6,620	6,940	94.3	3.14
23	20	157.5	81.25	56.0	1.55	2.24	87.6	78.7	83.2	8.9	6,760	7,120	101.0	2.58
24	22	165.0	84.0	47.5	1.5	1.97	81.2	73.6	77.4	7.6	6,500	6,820	92.9	2.8
25	23	172.5	91.5	52.25	1.61	2.12	82.5	73.6	78.1	8.9	7,150	7,550	88.0	3.32
26	22	168.5	83.5	51.0	1.57	2.09	83.8	74.9	79.4	8.9	6,630	7,000	95.7	2.98
27	21	175.0	89.5	56.25	1.68	2.24	82.5	77.5	80.0	5.0	7,160	7,380	92.2	3.26
28	20	166.5	87.25	53.75	1.59	2.17	82.5	74.9	78.7	7.6	6,870	7,200	93.2	3.36
29	18	170.25	91.0	60.0	1.75	2.34	85.1	76.8	82.0	8.3	7,460	7,740	92.7	3.48
30	21	168.25	86.5	48.25	1.53	2.00	80.6	73.6	77.1	7.0	6,670	6,970	90.7	3.52

31	22	172.75	88.75	59.5	1.70	2.32	85.1	75.5	86.3	9.0	7,120	7,550	91.8	3.18
32	22	163.0	88.5	59.0	1.59	2.24	90.1	78.7	84.2	11.1	7,060	7,520	93.1	3.21
33	21	168.0	80.5	44.5	1.48	1.89	76.2	68.6	72.4	7.6	6,260	6,590	86.3	2.51
34	20	171.5	89.5	55.0	1.63	2.20	86.3	78.7	82.5	7.6	7,380	7,720	91.5	2.90
35	21	172.5	88.0	54.0	1.63	2.17	83.2	76.2	81.7	7.0	7,190	7,320	92.5	3.72
36	22	170.25	90.25	52.5	1.6	2.14	88.9	78.7	83.8	10.2	7,560	8,020	80.4	3.86
37	21	163.5	84.0	60.0	1.63	2.34	88.9	81.3	85.1	7.0	7,160	7,470	100.0	2.32
38	21	168.0	89.0	52.5	1.57	2.14	85.1	74.9	80.0	10.2	7,120	7,570	90.6	3.98
39	18	166.75	85.0	53.25	1.58	2.16	79.7	71.1	75.2	8.6	6,390	6,770	95.3	2.6
40	20	169.75	90.0	54.0	1.61	2.17	86.3	76.2	81.3	10.1	7,320	7,770	90.5	4.10
41	23	168.0	87.0	44.75	1.46	1.90	86.3	74.9	80.6	11.1	7,010	7,510	87.9	2.6
42	21	170.0	88.25	40.0	1.5	1.94	76.2	72.4	74.0	3.8	6,530	6,720	87.5	2.60
43	21	163.0	85.75	67.25	1.72	2.55	93.9	86.3	90.1	7.6	7,770	8,050	102.0	2.94
44	21	170.75	88.25	54.5	1.62	2.18	87.0	78.7	82.9	8.3	7,320	7,690	92.6	3.24
45	24	180.5	91.25	55.0	1.7	2.20	86.1	75.5	80.8	10.6	7,370	7,850	80.8	3.54
46	21	167.5	89.25	56.75	1.49	2.25	87.6	78.7	83.2	8.9	7,010	7,820	92.7	3.86
47	20	163.5	83.5	47.5	1.48	1.97	82.5	76.8	79.7	5.7	6,080	6,890	93.4	2.0
48	20	160.0	84.75	55.75	1.56	2.22	88.9	78.7	83.8	10.2	7,100	7,530	97.1	2.8
49	20	169.5	87.0	52.25	1.59	2.14	86.3	77.4	81.9	8.9	7,120	7,510	92.6	3.14
50	22	166.75	85.25	55.5	1.58	2.21	88.9	80.0	84.5	8.9	7,200	7,580	96.4	2.2
51	22	178.75	91.5	59.0	1.74	2.32	87.6	80.0	83.8	7.6	7,920	8,280	88.8	2.38
52	20	164.5	85.25	57.5	1.61	2.26	83.8	76.2	80.0	7.6	6,820	7,140	97.5	2.38
53	19	177.0	87.75	64.5	1.78	2.48	85.7	76.2	81.0	9.5	7,110	7,520	98.4	3.46

TABLE I—*contd.*

Serial number	Age in years	Standing height, cm	Sitting height, cm	Weight, kg	Surface area (Dubois), sq m	Surface area (Dreyer), sq m	Chest circumference inflated, cm	Chest circumference deflated, cm	Chest circumference mean, cm	Chest expansion, cm	Trunk area (sitting height \times mean chest), sq cm	Trunk area (sitting height \times inflated chest), sq cm	Pelvis (von Parquet index)	Vital capacity, litres
54	20	162.5	83.0	56.25	1.59	2.24	86.3	78.7	82.5	7.6	6,850	7,160	99.4	2.64
55	20	172.0	85.5	51.0	1.59	2.09	82.5	73.6	78.1	8.9	6,680	7,050	93.4	3.4
56	18	173.0	88.0	58.25	1.69	2.20	85.7	76.2	81.0	9.5	7,130	7,540	94.9	3.62
57	20	172.75	84.75	65.5	1.77	2.50	85.7	78.1	81.9	7.6	6,040	7,260	102.4	3.14
58	19	171.25	82.5	55.25	1.64	2.21	85.1	76.2	80.7	8.9	6,660	7,020	99.4	3.36
59	20	169.5	87.0	49.75	1.56	2.04	81.9	73.6	76.8	8.3	6,680	7,120	91.0	2.6
60	21	173.5	88.5	56.0	1.66	2.24	82.5	73.7	78.1	8.8	6,010	7,300	93.1	3.4
61	18	169.0	90.0	66.75	1.76	2.51	83.8	78.7	81.3	5.1	7,320	7,540	97.1	2.46
62	20	173.5	88.5	51.0	1.60	2.09	78.7	73.7	76.2	5.0	6,740	6,960	90.2	2.82
63	21	163.75	87.25	46.0	1.47	1.93	76.2	68.6	72.4	7.6	6,320	6,650	88.4	2.76
64	23	170.0	86.75	54.25	1.61	2.16	78.7	72.4	75.6	6.3	6,560	6,830	94.0	2.44
65	22	149.0	79.5	44.25	1.35	1.88	77.5	70.5	74.0	7.0	5,880	6,160	95.8	2.6
66	23	169.0	82.25	57.25	1.65	2.25	80.3	77.5	81.9	8.8	6,740	7,100	100.9	3.42
67	23	161.5	88.5	52.5	1.54	2.14	81.3	73.7	77.5	7.6	6,800	7,190	91.1	3.38
68	21	177.0	90.0	50.25	1.72	2.32	83.8	76.2	80.0	7.6	7,200	7,540	93.3	3.56
69	21	171.5	86.5	52.25	1.60	2.14	83.8	72.4	78.1	11.4	6,700	7,250	93.1	2.92

70	21	172.5	86.0	70.25	1.82	2.02	91.4	65.1	88.3	0.3	7,000	7,800	103.3	2.34
71	22	167.0	85.75	40.75	1.50	1.97	78.7	70.5	74.6	8.2	6,400	6,750	90.5	2.8
72	24	160.25	84.75	48.5	1.48	2.01	90.0	73.0	76.5	7.0	6,480	6,780	92.7	2.08
73	22	168.5	84.0	57.75	1.03	2.28	82.6	70.2	79.4	6.4	6,070	6,910	90.1	2.46
74	25	172.75	91.0	60.5	1.71	2.36	83.8	70.2	80.0	7.0	7,280	7,620	92.9	3.32
75	10	161.0	84.25	47.75	1.47	1.99	70.4	72.4	75.0	7.0	6,300	6,690	92.7	2.08
76	20	164.5	90.0	70.25	1.83	2.77	104.1	07.8	100.0	0.3	6,080	6,370	101.5	3.22
77	21	161.75	84.0	47.75	1.47	1.90	81.3	72.4	70.0	8.0	6,460	6,830	93.0	2.02
78	23	163.5	84.0	40.75	1.30	1.78	74.3	60.7	70.5	7.6	5,920	6,240	88.2	2.74
79	21	177.75	88.75	64.75	1.80	2.48	88.3	78.7	83.5	9.6	7,410	7,840	97.4	4.3
80	21	179.0	95.75	68.0	1.85	2.56	88.9	78.7	83.8	10.2	8,020	8,510	91.8	4.18
81	24	165.75	80.5	50.0	1.04	2.32	83.8	77.5	80.7	0.3	6,080	7,250	96.9	3.16
82	21	176.25	80.75	54.25	1.66	2.16	81.3	72.4	70.9	8.9	6,070	7,050	94.0	3.2
83	22	173.0	87.75	47.25	1.55	1.97	70.4	69.8	74.0	9.0	6,540	6,970	88.7	2.06
84	18	161.0	86.0	52.75	1.54	2.14	82.5	74.9	78.7	7.6	6,770	7,100	93.0	3.12
85	19	163.0	81.0	44.0	1.43	1.87	81.3	73.0	77.2	8.3	6,250	6,580	93.0	2.06
86	22	157.0	80.0	52.0	1.50	2.13	83.8	70.8	80.3	7.0	6,420	6,700	100.5	2.7
87	10	171.5	88.0	46.5	1.53	1.95	78.7	71.7	74.9	7.6	6,590	6,930	88.0	2.96
88	23	169.5	80.5	65.5	1.74	2.50	64.6	88.9	91.8	5.7	8,210	8,470	97.0	2.88
89	21	169.5	80.0	57.0	1.64	2.26	83.8	70.2	80.0	7.6	6,880	7,210	96.4	2.48
90	22	167.5	88.0	47.75	1.51	1.90	82.5	71.1	70.8	11.4	6,700	7,200	88.8	3.12
91	23	167.0	85.0	50.0	1.55	2.06	85.1	70.8	81.0	8.3	6,880	7,230	93.3	2.90
92	18	178.0	93.0	55.75	1.99	2.23	83.8	72.4	78.1	11.4	7,260	7,700	88.4	3.14

TABLE I—*contd*

Serial number	Age in years	Standing height, cm	Sitting height, cm	Weight, kg	Surface area (Dubois), sq m	Surface area (Dreyer), sq m	Chest circumference inflated, cm	Chest circumference deflated, cm	Chest circumference mean, cm	Chest expansion, cm	Trunk area (sitting height \times mean chest), sq cm	Trunk area (sitting height \times inflated chest), sq cm	Peldisi (von Parquet index)	Vital capacity, litres
93	23	164.0	84.5	61.5	1.05	2.39	94.0	86.4	90.2	7.6	7,020	7,940	100.0	2.98
94	20	169.0	80.75	82.0	1.192	2.94	105.4	95.2	100.3	10.2	8,080	9,140	107.8	3.92
95	19	170.0	91.25	59.0	1.08	2.32	87.0	77.5	82.3	9.5	7,510	7,940	91.9	3.38
96	20	169.25	89.5	62.5	1.71	2.41	90.2	80.0	85.1	10.2	7,610	8,070	95.5	3.02
97	26	157.5	81.0	60.25	1.67	2.52	94.0	83.8	88.9	10.2	7,200	7,610	107.0	3.0
98	20	159.5	83.0	46.50	1.45	1.95	78.7	69.9	74.3	8.8	6,170	6,530	93.3	2.28
99	21	159.5	80.0	41.75	1.39	1.80	76.2	68.6	72.4	7.6	5,790	6,100	93.4	2.08
100	22	154.5	81.5	45.75	1.41	1.93	78.7	72.4	75.5	6.3	6,150	6,410	94.5	1.96
101	20	174.0	89.0	51.25	1.61	2.19	83.8	76.8	80.3	7.0	7,150	7,460	89.9	3.26
102	19	170.5	87.75	59.5	1.69	2.32	90.2	80.0	85.1	10.2	7,470	7,610	95.8	3.7
103	21	166.5	85.0	52.0	1.57	2.11	88.9	77.5	83.2	11.4	7,070	7,560	94.0	2.24
104	21	164.0	86.25	54.25	1.59	2.16	87.6	78.7	83.2	8.9	7,170	7,550	94.5	3.1
105	19	169.0	87.75	52.25	1.58	2.12	87.0	80.0	83.5	7.0	7,330	7,630	91.7	3.14
106	22	177.0	87.0	63.5	1.78	2.44	88.9	82.6	85.8	6.3	7,460	7,730	98.7	3.36
107	21	168.0	85.75	52.25	1.58	2.12	85.0	76.8	80.9	8.2	6,940	7,290	93.9	2.94
108	20	179.5	95.25	62.25	1.77	2.40	91.4	83.8	87.6	7.6	8,340	8,700	89.6	3.4

109	20	107.75	89.75	64.5	1.71	2.47	01.4	84.4	87.9	7.0	7,890	8,200	90.2	2.06
110	21	154.25	79.75	40.9	1.45	2.03	83.8	70.2	80.0	7.0	6,380	6,680	98.8	2.00
111	21	107.0	86.0	52.0	1.56	2.11	82.5	74.9	78.7	7.0	6,770	7,100	93.5	2.84
112	23	158.5	80.5	56.25	1.56	2.24	80.3	78.7	82.5	7.0	6,040	6,950	102.5	2.74
113	22	160.5	87.0	50.5	1.05	2.32	90.2	80.0	85.1	10.2	7,400	7,850	96.0	2.02
114	22	161.5	81.0	51.25	1.52	2.19	79.3	74.9	77.1	4.4	6,250	6,420	98.8	2.7
115	27	174.5	90.0	49.75	1.09	2.05	77.5	68.0	73.1	8.9	6,580	6,970	88.0	3.02
116	22	161.5	86.0	49.5	1.50	2.04	77.5	72.4	75.0	5.1	6,450	6,600	91.9	2.64
117	22	160.0	82.25	52.75	1.53	2.14	87.0	77.5	82.0	10.1	6,790	7,200	98.2	3.02
118	22	160.0	82.75	65.25	1.71	2.48	80.4	78.7	82.5	7.7	6,830	7,150	104.8	2.74
119	22	164.5	85.0	48.0	1.56	1.99	83.2	72.4	77.8	10.8	6,010	7,070	92.1	3.3
120	20	165.0	83.0	51.75	1.55	2.10	82.5	74.9	78.7	7.0	6,530	6,850	90.7	3.04
121	20	171.0	90.0	70.0	1.8	2.62	94.0	83.8	80.2	10.8	8,030	8,510	98.0	3.8
122	21	161.0	82.5	58.25	1.69	2.29	88.9	79.4	84.1	9.5	6,940	7,330	101.2	3.3
123	20	169.25	84.75	55.75	1.63	2.23	87.0	78.7	83.2	8.9	7,650	7,420	97.1	3.02
124	22	161.75	82.0	40.0	1.45	1.98	83.8	73.7	78.7	10.1	6,450	6,870	94.1	3.12
125	22	165.75	86.0	60.75	1.66	2.30	80.3	78.7	82.5	7.0	7,100	7,420	98.4	3.54
126	23	165.75	87.5	59.75	1.65	2.33	85.7	80.0	83.2	5.1	7,280	7,560	96.2	2.96
127	24	167.5	86.5	52.75	1.58	2.14	82.5	74.9	78.7	7.0	6,810	7,100	93.4	2.72
128	23	161.5	84.0	55.75	1.57	2.23	89.5	80.0	84.8	9.5	7,120	7,520	97.9	3.08
129	20	155.25	82.0	44.0	1.39	1.88	78.1	72.4	75.3	5.7	6,170	6,400	92.7	2.7
130	21	169.25	89.0	48.0	1.53	1.99	81.3	73.7	77.5	7.0	6,960	7,240	87.9	2.68
131	22	156.75	82.75	50.25	1.55	2.24	88.9	82.5	85.7	6.4	7,090	7,350	99.7	2.02

TABLE I—*concd*

Serial number	Age in years	Standing height, cm	Sitting height, cm	Weight, kg	Surface area (Dubois), sq m	Surface area (Dreyer), sq m	Chest circumference inflated, cm	Chest circumference deflated, cm	Chest circumference mean, cm	Chest expansion, cm	Trunk area (sitting height \times mean chest), sq cm	Trunk area (sitting height \times inflated chest), sq cm	Pelidisi (von Pirquet index)	Vital capacity, litres
132	20	171.0	89.0	58.75	1.68	2.31	86.4	80.0	83.2	6.4	7,400	7,690	94.1	3.42
133	24	161.75	82.75	51.25	1.52	2.19	87.6	81.3	84.5	6.3	6,990	7,250	96.7	2.64
134	21	165.5	86.0	64.5	1.7	2.47	92.7	82.6	87.6	10.1	7,530	7,970	100.4	2.78
135	21	167.0	86.5	60.75	1.74	2.51	87.6	81.3	84.5	6.3	7,310	7,580	101.0	2.32
136	24	163.5	85.5	47.0	1.48	1.96	80.0	71.8	75.9	8.2	6,490	6,840	90.9	2.96
137	22	161.0	89.5	40.75	1.46	1.97	80.0	72.4	76.2	7.6	6,820	7,160	86.7	2.36
138	18	175.75	89.5	54.5	1.66	2.18	82.6	73.0	77.8	9.6	6,960	7,390	91.2	2.84
139	18	170.75	85.0	51.75	1.58	2.10	81.9	74.9	78.4	7.0	6,660	6,960	94.4	2.68
140	20	173.5	87.5	52.5	1.61	2.14	85.1	74.9	80.0	10.2	7,000	7,440	92.1	3.04
141	23	165.25	84.75	48.75	1.51	2.01	84.5	74.9	79.7	9.6	6,750	7,160	93.0	2.3
142	23	166.0	87.5	53.0	1.57	2.14	85.1	77.5	81.3	7.6	7,110	7,440	92.4	2.74
143	21	159.25	83.5	50.5	1.50	2.06	85.1	76.2	80.7	8.9	6,740	7,100	95.3	2.66
144	20	160.5	85.25	47.0	1.96	1.93	81.9	73.7	77.8	8.2	6,030	6,980	91.2	2.46
145	25	179.75	94.25	88.75	2.08	3.11	102.9	94.0	98.5	8.0	9,280	9,700	101.0	3.3
146	25	164.5	85.75	50.0	1.59	2.23	87.0	77.5	82.3	9.5	7,060	7,460	96.1	2.68
147	22	166.0	89.0	66.25	1.72	2.52	91.4	80.0	85.7	11.4	7,930	8,130	97.9	3.40
148	22	160.0	83.0	56.25	1.57	2.24	84.5	70.8	80.6	7.7	6,690	7,010	99.4	2.6

119	23	171.5	80.5	51.0	1.00	2.09	83.8	75.5	79.7	8.3	7,130	7,500	89.2	2.9
120	21	168.5	85.75	56.75	1.04	2.25	83.8	76.2	80.0	7.0	6,800	7,180	96.5	2.7
121	22	162.5	84.0	45.25	1.44	1.91	83.8	74.9	79.4	8.9	6,070	7,040	91.3	3.2
122	20	167.25	87.5	71.75	1.78	2.06	90.5	88.9	92.7	7.0	8,110	8,140	102.3	2.4
123	20	168.25	80.75	52.5	1.60	2.14	81.9	76.2	79.1	5.7	6,860	7,100	92.9	2.7
124	21	172.75	88.25	59.75	1.70	2.33	87.0	78.7	82.9	8.3	7,120	7,080	95.4	3.34
125	20	163.75	80.0	60.0	1.09	2.34	80.5	81.0	85.3	8.5	7,330	7,700	98.0	2.8
126	20	167.0	80.0	62.25	1.57	2.12	80.0	73.7	76.9	6.3	6,610	6,880	93.6	2.04
127	20	163.5	84.5	40.75	1.48	1.97	81.3	74.3	77.5	7.0	6,570	6,870	91.8	2.28
128	21	168.5	85.25	52.5	1.59	2.14	83.8	74.3	79.1	9.5	6,740	7,140	94.0	2.92
129	21	150.0	81.0	43.5	1.41	1.80	80.0	69.2	74.6	10.8	6,040	6,480	93.5	2.3
130	20	161.5	85.5	47.25	1.47	1.97	81.3	72.4	79.9	8.9	6,570	6,950	91.0	2.5
131	24	177.25	90.5	62.5	1.77	2.41	91.4	82.6	87.0	8.8	7,570	8,270	94.4	2.76
132	21	167.25	87.0	50.5	1.55	2.07	81.3	72.4	79.0	8.9	6,090	7,070	91.5	2.58
133	21	161.5	80.25	50.25	1.51	2.06	80.0	71.1	75.6	8.9	6,070	6,420	90.0	2.94
134	18	167.5	87.25	42.75	1.44	1.83	73.7	60.0	69.9	7.7	6,100	6,430	86.3	2.1
135	20	164.5	88.0	50.5	1.64	2.32	90.2	78.7	84.5	11.5	7,440	7,940	95.5	1.9
136	21	165.5	86.0	55.0	1.59	2.20	83.8	74.9	79.4	8.9	6,840	7,210	95.2	2.74
137	21	172.5	88.5	50.25	1.69	2.32	80.4	77.5	82.0	8.9	7,200	7,640	94.9	3.5
138	23	160.5	82.0	49.0	1.48	2.03	79.4	72.4	75.9	7.0	6,220	6,510	90.1	2.6
139	20	160.75	87.5	40.75	1.45	1.97	79.4	72.4	75.9	7.0	6,640	6,950	88.0	2.14
140	21	158.75	85.0	55.0	1.55	2.20	85.1	70.2	80.7	8.9	6,860	7,230	96.3	2.08
141	21	154.5	81.0	45.25	1.4	1.91	85.7	74.9	80.3	10.8	6,590	6,940	94.7	2.6
142	20	160.75	86.5	58.75	1.64	2.31	83.8	70.2	80.0	7.6	6,920	7,250	96.8	2.52

TABLE II

Statistical distribution of data

Number of subjects 172 Sex Male

Measurement	Range	Mean \pm P E _m	Standard deviation, $\sigma \pm$ P E _{σ}	Coefficient of variation, per cent \pm P E _{c v}
Age in years	18-29	21.8 \pm 0.0835	1.72 \pm 0.0625	7.89 \pm 0.294
✓ Standing height, cm	149-179.75	167.08 \pm 0.311	6.06 \pm 0.2204	3.63 \pm 0.135
✓ Sitting height, cm	79.5-95.75	86.58 \pm 0.164	3.18 \pm 0.116	3.67 \pm 0.137
✓ Weight, kg	41.75-97	55.6 \pm 0.445	8.64 \pm 0.314	15.54 \pm 0.579
Surface area (Dubois), sq m	1.35-2.08	1.606 \pm 0.0063	0.1224 \pm 0.0044	7.62 \pm 0.284
Surface area (Dreyer), sq m	1.78-3.31	2.2 \pm 0.0126	0.246 \pm 0.0089	11.18 \pm 0.417
Chest inflated, cm	73.7-105.4	85.12 \pm 0.282	5.48 \pm 0.199	6.44 \pm 0.240
Chest deflated, cm	66-97.8	76.52 \pm 0.268	5.2 \pm 0.189	6.79 \pm 0.253
Chest mean, cm	69.9-100.9	80.88 \pm 0.268	5.2 \pm 0.189	6.43 \pm 0.240
✓ Chest expansion, cm	3.8-11.5	8.26 \pm 0.0812	1.576 \pm 0.0573	19.08 \pm 0.711
Trunk area—sitting height \times mean chest, sq cm	5790-9280	6986.4 \pm 30.66	596 \pm 21.66	8.53 \pm 0.318
Trunk area—sitting height \times inflated chest, sq cm	6100-9700	7342 \pm 31.59	614 \pm 22.33	8.36 \pm 0.312
Pelldisi (von Pirquet index)	86.3-107.8	95.22 \pm 0.222	4.32 \pm 0.157	4.54 \pm 0.169
✓ Vital capacity (litres)	1.96-4.3	2.949 \pm 0.0242	0.471 \pm 0.0171	15.97 \pm 0.595

TABLE III

Statistical correlation of vital capacity and other measurements

Correlated data	Correlation coefficient $r \pm P E_r$	Correlation ratio, $\eta \pm P F_\eta$	$\eta^2 - r^2 (z) \pm P E_z$
<i>Vital capacity and—</i>			
Trunk area (sitting height × inflated chest)	+ 0.47 ± 0.0403	0.518 ± 0.0378	0.0474 ± 0.021
Sitting height ✓	+ 0.455 ± 0.041	0.489 ± 0.0394	0.0321 ± 0.0175
Standing height ✓	+ 0.44 ± 0.0417	0.453 ± 0.0411	0.0116 ± 0.0107
Trunk area (sitting height × mean chest)	+ 0.413 ± 0.0429	0.49 ± 0.0393	0.0695 ± 0.0248
Surface area (Dubois) ✓	+ 0.395 ± 0.0437	0.449 ± 0.0413	0.0455 ± 0.0205
Chest circumference inflated	+ 0.369 ± 0.0447	0.413 ± 0.0429	0.0344 ± 0.018
Weight	+ 0.352 ± 0.0453	0.425 ± 0.0424	0.0567 ± 0.0226
Chest expansion ✓	+ 0.335 ± 0.0459	0.385 ± 0.0441	0.036 ± 0.0184
Chest circumference mean	+ 0.314 ± 0.0466	0.387 ± 0.044	0.0511 ± 0.0216
Surface area (Dreyer)	+ 0.307 ± 0.0469	0.407 ± 0.0432	0.0714 ± 0.025
Chest circumference deflated	+ 0.258 ± 0.0483	0.358 ± 0.0451	0.0616 ± 0.0234
Age	- 0.018 ± 0.0517	0.127 ± 0.0509	0.0158 ± 0.0124
Pelldisi	+ 0.003 ± 0.0516	0.237 ± 0.0488	0.0561 ± 0.0224

TABLE IV

Regression equations of vital capacity and other measurements

1	Vital capacity (litres)	=	0.0342	standing height, cm	-2.765
2	"	"	"	=	0.0665 sitting height, cm -2.81
3	"	"	"	=	0.019 weight, kg + 1.8936
4	"	"	"	=	1.517 surface area, Dnbois, sq m + 0.514
5	"	"	"	=	0.587 surface area, Dreyer, sq m + 1.66
6	"	"	"	=	0.0317 chest circumference inflated, cm + 0.25
7	"	"	"	=	0.0233 chest circumference deflated, cm + 1.169
8	"	"	"	=	0.0284 chest circumference mean, cm + 0.655
9	"	"	"	=	0.1 chest expansion, cm + 2.124
10	"	"	"	=	0.00033 trunk area (sitting height, cm × mean chest circumference, cm), sq cm + 0.67
11	"	"	"	=	0.00036 trunk area (sitting height, cm × inflated chest circumference, cm), sq cm + 0.307

TABLE V

Vital capacity figures for Indians

Workers	Number of subjects	Age in years	Vital capacity, c.c.	Vital capacity, c.c. Standing height, cm	Vital capacity, c.c. Surface area, Dubois, sq m	Province
1 Bhatia (1929)	100	20-45	3,096	18.52	1,960	Bombay
2 Krishnan and Vareed (1932)	103	18-29	2,929	17.5	1,850	Madras
3 Krishnan and Vareed (1933)	198	17-26	3,050	18.5	1,930	"
4 De and De (1939)	100	17-23	2,721	16.5	1,790	Bengal
5 Telang and Bhagwat (present communication)	172	18-29	2,949	17.65	1,830	Bombay
AVERAGE			2,949	17.73	1,870	India

TABLE VI

Vital capacity figures for non-Indians

Workers	Number of subjects	Age in years	Vital capacity, c.c.	Vital capacity, c.c. Standing height, cm	Vital capacity, c.c. Surface area, sq. m.	Nationality
1 Foster and Hseih (1923)	425	Adult	3 180	19 50	2,020	Chinese
2 Satake and Sato (1938)	230	19-26	3 800	23 3	2,380	Japanese
3 Hutchinson (1846)	1,285		3,602	21 0	2,010	English
4 Schuster (1911)	959	18-23	4 315	24 5	2 350	,
5 Drever (1919)	16	13-52	4,140	24 4	2 400	„
6 Cripps Greenwood and Newbold (1923)	1 235	(Mean) 23 92	4 569	26 1	2 600	„
7 Cripps Greenwood and Newbold (1923)	959	(Mean) 23 80	4 604	26 4	2,600	„
8 Hobson (quoted by Mumford and Young 1923)	100	Adult	4 823	26 9	2,580	„
English average			4 342	24 9	2 420	„
9 West (1920)	85		4 651	26 8	2,610	American
10 Hewlett and Jackson (1922)	400	18-30	4,646	26 4	2,530	„
11 Lemon and Moersh (1924)	165	19-70	4 487	26 2	2 490	„
12 Jackson and Lees (1929)	100	17-22	4 406	25 5	2 550	„
American average			4,547	26 2	2,540	,

TABLE VII

Proportional comparison of Indian and non-Indian figures for vital capacity

	INDIAN AVERAGE FORMS PER CENT OF				
	Chinese average (selected cases ?), per cent	Japanese average (selected cases ?), per cent	English average (selected and random cases together), per cent	Hutchinson's average (random cases), per cent.	American average (selected cases ?), per cent
Vital capacity	93	77.5	68	82	65
$\frac{\text{Vital capacity, c.c.}}{\text{Standing height, cm}}$	91	76	71	84	68
$\frac{\text{Vital capacity, c.c.}}{\text{Surface area, Dubois, sq. m.}}$	92	78.5	77	93	74

TABLE VIII.

Comparison of standing height and body surface area of Indians and non-Indians

Nationality	Average standing height, cm	Average body surface area, Dubois, sq. m.
Indian	167	1.58
Chinese	163	1.57
Japanese	163	1.59
English	174	1.79
American	173.5	1.79

METALLIC CONTAMINATION OF FOODSTUFFS

Part IV

INVESTIGATIONS ON CONTAMINATION OF FOODSTUFFS WITH COPPER FROM BRASS AND BRONZE VESSELS AND THE METABOLIC EXPERIMENTS ON THE ABSORPTION AND EXCRETION OF COPPER IN RATS

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COPPER is present in all living matter. Marine animals are known to contain copper in considerable amounts. Bodansky (1921) found copper in adult brain to the extent of 3.6 mg to 6.0 mg per kg. Morrison and Nash (1930) showed that the livers of infants contain about 24 mg of copper per kilo fresh tissue, while adult livers contain only 4 mg. Warburg and Krebs (1927) reported small but definite amount of copper in human blood serum. The copper content of blood has been studied by several workers. The results of different workers are given in a tabular form by Tompsett (1934). Most of the values for the copper content of 100 c.c. of whole blood was found to lie between 0.05 mg and 0.25 mg.

It has now been proved beyond doubt that copper as a supplement to iron is essential in the regeneration of haemoglobin in blood. The exact mechanism by which copper acts in biological processes is, however, not known. The recent investigations on the copper activity deals with copper content of different tissues, the change in the copper content of blood and its effect on the storage and utilization of iron.

Copper in foods — On the light of our present knowledge copper may be regarded as an essential element in nutrition and, therefore, the supply of this element

in normal foodstuffs is also important. The copper content of foodstuffs have been studied by a number of workers. Greens and Anderson (1935) have analysed the copper content of a large number of samples of wheat, barley and peas and observed that copper content varied from 6.2 to 11.2 parts per million.

Copper content of milk has received considerable attention. Supplee and Bellis (1922) found the copper content of cow's milk on an average to be 0.52 mg per litre. Hess, Supplee and Bellis (1923) reported that human milk on a mixed diet contain copper varying from 0.4 mg to 0.6 mg per litre. Conn *et al* (1934) reviewed much of the work on the copper content of milk and found it to vary from 0.051 to 0.132 parts per million.

It appears, therefore, that copper is a constant constituent of all foodstuffs whether of animal or vegetable origin and naturally both infants and adults are continually ingesting copper in their daily diet. A part of the copper is absorbed from the alimentary canal as proved from its constant presence in urine. Robinowitch (1933) found that the copper content of 24 hours' urine ranges between traces and 0.7 mg.

Copper added to food as a result of contamination from vessels and utensils — The amount of copper present in natural foodstuffs is very small but may increase to a considerable extent as a result of contamination. Brass, bronze and tinned brass vessels which are largely used in different parts of India are undoubtedly the source of contamination of food with copper. Brass and bronze vessels are used throughout India. Food and drinks are served on plates and tumblers made of bronze. Brass vessels are used for cooking rice, *dhal* and vegetables in many houses in India. Brass cups are largely used for serving tea and coffee throughout South India. Brass vessels are the common containers used for transport and distribution of milk in villages, towns and in cities. Tinned brass vessels, which are very popular in the South, also serve as source of copper if re-tinning is not done in proper time after long use.

Copper within certain limiting concentration may perform certain important physiological function in the body, whereas larger quantities that enter food as a result of contamination may exert harmful effect on the system. It is known that copper is toxic in large doses. Mallory and Parker (1931) have attempted to show that copper in food due to the use of copper vessels is the cause of the disease known as hæmochromatosis. In a recent review on hæmochromatosis Sheldon (1934) states that it is difficult to accept the view that the disease is produced by copper poisoning.

As to the toxicity of metal there still prevails an uncertainty owing to the contradictory evidence in the literature. There is a real need for information as to whether there is a *minimum* for the metal above which it is dangerous to go and what does the body do with the excess. Hughes (1935) suggested that some definite and unbiased opinion as to the safety limit of metals like copper and tin in food is desirable. This is useful for the public as users of household metallic utensils and containers.

In the present preliminary investigation attempts have been made to get an idea as to the amount of copper normally present in common Indian foodstuffs and the quantity of copper that is added to the food from the vessels, to find out the mode of excretion of this metal from the body with different types of food and finally its effect on the growth and well-being of the animals

EXPERIMENTAL

Copper content of Indian foodstuffs—Copper is essential in nutrition. The daily requirement of a normal human being is about 2 mg per day. Damels and Wright (1934) suggest that the diet of pre-school children should contain copper not less than 0.1 mg per kilo body-weight. Hodges and Peterson (1931) calculated the daily copper intake for sixteen different menus and found that the figures range from 0.8 mg for a child to 4.81 mg for the working man. In order to find out whether the average Indian diet supplies the daily requirement of this element, a knowledge as to the quantity of this metal in common foodstuffs is desirable.

TABLE I

The copper content of some of the Indian foodstuffs

(Determined by the method of Biazzo, 1926)

Foodstuffs	Copper as p.p.m. on dry weight basis	Foodstuffs	Copper as p.p.m. on dry weight basis
Milk	0.25	Cauliflower	4.73
Wheat, sample I	4.03	Carrot	5.88
Wheat sample II	2.73	Brinjal	10.64
Bengal gram (<i>dhal</i>)	4.23	Green <i>dhal</i>	12.50
Musur <i>dhal</i>	4.23		

The estimation of copper in the foodstuffs in Table I was carried out by the application of Biazzo's (1926) method. In the subsequent determinations the method adopted by Sylvester and Lampitt (1935) was followed. The method is based upon the quantitative extraction of copper from an acid solution by means of chloroform solution of diphenyl thiocarbazon. After removal of chloroform, the residue containing the whole of copper is digested with sulphuric acid and

perchloric acid and copper is determined colorimetrically by means of sodium-diethyl-dithiocarbamate

TABLE II

The copper content of some of the Indian foodstuffs

(Determined by the method of Sylvester and Lampitt, 1935)

Foodstuffs	Copper as ppm on dry weight basis	Foodstuffs	Copper as ppm on dry weight basis
Rice, polished	2.9	Musur dhal	6.9
Wheat (bazaar sample)	6.4	Beans	7.2
Bengal gram	6.0	Carrot	10.6
Horse gram	10.6	Cabbage	10.0
Green dhal	11.6	Black gram	8.6
Milk	0.18		

THE CONTAMINATION OF FOODSTUFFS WITH COPPER FROM BRASS AND BRONZE VESSELS

The effect of boiling and storage of milk in brass and bronze vessels—The amount of copper in uncontaminated raw milk is important from physiological point of view. Another aspect of the problem, namely, the amount of copper added to milk from the vessels and the effect it produces on the milk itself, also requires investigation. The presence of copper in milk is known to bring about certain typical changes in the milk, especially off-flavour. Guthrie, Roadhouse and Richardson (1931) kept thin strips of different metals in contact with sweet milk in order to find out the suitability of these metals for milk equipment and observed that copper alloys like brass and bronze showed weight losses and produced oxidized flavour in the milk. Tin-plated copper was also considered unsuitable because of the mechanical wearing away of the plating. Hess and Unger (1921-22) have shown that the vitamin C content of milk is greatly reduced when milk is contaminated with copper. Schwartze, Murphy and Gerald (1931) observed that the vitamin C content of milk is destroyed from 80 to 90 per cent when milk is pasteurized at 60°C in copper pasteurizer for 30 minutes, while with glass or aluminium the destruction is only 20 to 40 per cent. The presence of copper acts as a catalyst in the destruction of vitamin C. Besides these changes, the excess of copper in milk may produce

toxic effect in the body. It seemed necessary, therefore, to estimate the copper content of milk and milk products boiled or stored in these vessels for different lengths of time.

TABLE III

The copper content of milk and curds boiled and stored in brass and bronze vessels

Nature of material	Treatment	Vessel used	Time of storage in hours	Copper as p.p.m.
Raw milk (100 c.c.)	Stored	Brass	4	10.3
	Boiled and stored		1	10.0
	"	"	4	17.5
	"	"	20	44.0
Milk (100 c.c.)	Stored	Bronze	24	0.5
Curd (50 c.c.)	"	"	24	12.4
	"	Brass	24	36.2

From the result of Table III, it is apparent that in the process of boiling or of transport of milk in brass containers for distribution, a quantity of copper is added to food material. The extent to which milk or milk products are contaminated with the metal depends on the duration of boiling or of storage. Prolonged storage of milk in brass vessels produces a bluish tint and a metallic taste and should always be avoided. With the superior quality of bronze vessel used in the experiment the contamination of milk was only very slight.

TABLE IV

The copper content of neutral foodstuffs like dhal and vegetables cooked in brass vessels

Foodstuffs	Copper dissolved in mg
50 g. of cabbage boiled with 500 c.c. of water and evaporated to 50 c.c.	3.0
30 g. musur dhal boiled for 1 hour with 250 c.c. of water	6.5

The result of Table IV clearly shows that neutral foods like *dhal* and vegetables when cooked even without salt in brass vessels are contaminated with copper to a considerable extent

TABLE V

Copper content of foodstuffs stored in bronze vessels of different composition

		COMPOSITION OF BRONZE IN			
		Vessel 1		Vessel 2	
		Cu and Sn 80 20	(as per cent)	Cu and Sn 85 15	
Foodstuffs	Duration of storage (hour)	Copper as p p m			
Milk	6	16.5		13.4	
Curd	24	9.0		27.5	
Rasam	6	15.6		25.8	
Orange juice	5	41.5		71.25	
Grape juice	6	61.25		131.25	
Butter milk	20	41.3		115.0	

Both the vessels employed in this experiment had been previously used in the household. It is evident from the results of Table V that contamination of foods with copper will depend largely on the composition of the alloy used in the construction of the vessels. This is particularly marked with acid foods. Although highly acid foods are rarely stored in brass or bronze vessels, the high copper content of rasam, grape juice and butter-milk stored in bronze vessels, points to the danger of storing such foods in tinned brass or bronze vessels with poor tinning or when re-tinning is not done in proper time.

METABOLIC EXPERIMENT ON THE ABSORPTION AND EXCRETION OF COPPER IN RATS

The type of diet is very important in determining the level of copper which is toxic. Coulson *et al* (1934) suggest that the amount of copper assimilated depends on the other constituent of the diet. Toxicity of the metals also depends on the mode of excretion of the metal from the body. In the present investigation

attempts have been made to follow the excretion of copper in the urine and faeces of rats with copper added with different diet and also from food contaminated with the metal from vessels during cooking or storage. Nine mg of copper as copper sulphate were thoroughly mixed with the wheat and was given to each rat in the form of wheat *chapatti* together with 1 c.c. of butter fat. Three rats used in this experiment were kept in separate metabolic cages made of glass. The urine and faeces were collected separately. Analysis for the copper content was carried out in every 2 days' sample of faeces and in the urine collected during the course of about a week. The quantity of food left over was carefully collected and analysed for copper and the values were deducted from the amount of copper added to the diet of each rat. The result of analyses of copper excretion conducted on 3 animals during the whole course of the experiment are recorded in Table VI.

TABLE VI

The quantity of copper excreted in the faeces and urine of rats kept on wheat chapatti with copper added as copper sulphate

Rat I

Copper intake in mg in 2 days	COPPER EXCRETION IN MG	
	Faeces in 2 days' sample	Urine
2.67	0.35	0.11 in 2 days
7.25	5.20	0.34 in 8
7.00	6.03	
9.85	8.92	
9.08	7.50	
9.90	7.50	0.20 in 6
9.18	10.27	
7.10	8.75	
No copper	4.25	0.08 in 4
"	1.20	
Total copper intake 62.03	Total excretion in faeces 59.97	Total excretion in urine 0.71

TABLE VI—concl'd
Average of rats II and III

Copper intake in mg in 2 days	COPPER EXCRETION IN MG	
	Fæces in 2 days	Urine
7.75	4.47	0.09 in 2 days
6.90	1.75	0.20 in 8 "
5.50	4.90	
13.00	7.50	
8.61	11.80	
9.75	8.25	0.14 in 6 "
9.50	8.75	
5.50	8.10	
No copper	3.92	0.09 in 4 "
" "	1.11	
Total copper intake 66.53	Total excretion in fæces 62.45	Total excretion in urine 0.52

The result of Table VI shows that 94 to 96 per cent of copper given to rats as copper sulphate mixed with wheat is excreted in the fæces, the amount appearing in the urine being only 0.8 to 1 per cent. The amount of copper unaccounted for, being 3 to 4 per cent, may be retained in the body.

Investigation on the excretion of copper added to milk—To each of the 4 rats kept in separate metabolic cages were given 25 c.c. of milk to which were added 3 mg. of copper as copper sulphate. The urine and fæces were collected and analysed for copper as before.

TABLE VII

The average excretion of copper in urine and faeces of 4 rats, each receiving 25 c c of milk to which were added 3 mg copper as copper sulphate

Days	Copper intake per rat in mg	COPPER EXCRETION PER RAT IN MG	
		Faeces	Urine
1st	3.0	0.40	0.02
2nd	3.0	1.24	0.02
3rd and 4th	6.0	4.65	0.035
5th and 6th	6.0	4.92	0.04
7th to 10th	No copper	5.77	0.072
Total copper intake 18.0		Total excretion in faeces 16.98	Total excretion in urine 0.187

94.5 per cent of the added copper was excreted in the faeces and only about 1 per cent in the urine. Three to four per cent of the copper remained unaccounted for as in the previous case.

Investigation on the excretion of copper in rat kept on milk stored in brass vessels — Fresh milk was boiled in a brass vessel and then stored in the same vessel for about 24 hours. Thirty c c of the stored milk was then given to each of the 4 rats kept in separate metabolic cages made of glass. Urine and faeces were collected as usual and analysed for copper. The results are included in Table VIII —

TABLE VIII

*The average excretion of copper in urine and faeces of 4 rats
Each rat was given 30 c c of milk boiled and
stored in a brass vessel*

Days	Copper intake per rat in mg	COPPER EXCRETION PER RAT IN MG	
		Faeces	Urine
4th	10.4	5.97	0.1
4th	No copper	3.41	0.17
Total copper intake 10.4		Total excretion in faeces 9.38	Total excretion in urine 0.17

About 90 per cent of the copper from contaminated milk was excreted in the faeces and only 1.66 per cent appeared in the urine. The amount retained in the body may be from 6 to 7 per cent. The results indicate a slightly higher excretion in the urine and increased retention in the body as compared with the result of Table VII, where copper is added to the milk as copper sulphate. The difference though slight may be explained by the fact that copper sulphate added to milk is precipitated as copper albuminate, whereas in the case of contaminated milk there is no visible precipitation and hence the metal may remain in a form in which it is more available for absorption. This point requires further investigation.

The toxic effect of copper as determined by the growth of animals—Copper in moderate doses produces no ill effect but is highly toxic in large doses. We have no definite knowledge as to the limit of copper in foodstuffs beyond which it is toxic. In determining the harmful effect of copper in foods, the nature and the constituents of the diet play an important part. Waddell, Steenboch and Hart (1931) observed that 4 mg of copper as copper sulphate given to each rat daily along with milk proves injurious but the same quantity when given along with mixed diet, the animals grow normally. The present studies are only a preliminary attempt to get an idea as to the limiting value for copper in foods beyond which it may prove toxic.

Young albino rats weighing about 35 g to 45 g were divided into three groups. Each rat was given wheat *chapatti* and 10 c.c. milk. Four mg, 6 mg and 9 mg of copper as copper sulphate were added to the milk and given to each of the rats placed in groups I, II and III respectively. A number of animals were kept as a control without any copper added to the milk.

TABLE IX

Growth of rats during 60 days of experiment, with different levels of copper as copper sulphate added to milk

Group	Copper given to each rat in mg	Number of rats	Sex	AVERAGE WEIGHT IN GRAMMES		
				Original	Final	Increase
I	4	8	♂	40	111	71
		4	♀	38	95	57
II	6	10	♂	40	90	50
III	9	6	♂	42	92	50
Control	Nil	15	♂	44	118	74
		4	♀	38	99	61

The results of Table IX clearly show that the growth of rats of group I do not differ significantly from those of the controls, the animals appeared quite normal and active. The animals of groups II and III (given 6 mg and 9 mg of copper as copper sulphate along with milk) grew at a distinctly lower rate than the controls. The rats appeared less active, the hair coat slightly pigmented and coarse as compared to those of the controls which were smooth and perfectly white.

DISCUSSION

Small quantities of copper are present in natural foodstuffs. Large quantities may, however, gain access to the food as a result of contamination from brass, bronze and tinned brass vessels which are extensively used throughout India. The extent of contamination of foodstuffs from vessels will depend on the nature of the food, the time of exposure of the metal to the food and to a large extent on the composition of metals or alloys used in the construction of vessels. Whether the quantities of copper that are added to the food from the vessels exert any harmful effect on those who consume it still remains an unsolved problem. It is evident from the results of metabolic experiments on copper in rats as shown in Tables VI and VII that 94 to 96 per cent of copper added either to wheat or to milk is excreted in the faeces but only a very small part is absorbed as indicated by the presence of slight but constant quantity of copper in urine. The amount of copper that enters food as a result of contamination is metabolized in a manner very similar to that of added copper except in that it shows a slightly higher excretion in urine and slight increased retention in the body. There are evidences to show that small quantities of copper added to the diet are absorbed and deposited in different organs of the body. It is not impossible, therefore, that small quantities of copper that are added to the food from the vessels, if taken in regularly for a fairly long time, may be deposited in different organs and exert their harmful effect.

Feeding experiments extending over longer periods and if possible on successive generations of animals have been arranged in order to study the effect on the growth and general well-being of the animals and also to find out if any abnormal quantities of the metals are deposited in such organs as liver and kidney as may produce degenerative changes in the cell structure. The investigation will also include the study of the effect of the traces of metals deposited on the enzyme system present in those organs.

In the absence of definite evidence, great caution should be exercised in the use of copper, brass and bronze vessels. Tinned brass vessels should have a uniform coating of tin. Although gross contamination with copper is rare, yet it is well to keep in mind that traces of copper that finds their way into the food from the vessels may prove harmful in the long run.

SUMMARY

The copper content of some of the Indian foodstuffs ranges from 0.18 parts per million in milk to 12.5 parts per million in cereals.

2 The quantities of copper that enter food as a result of contamination from vessels depend on the nature of food, the time of exposure of the food to the metal and to a large extent on the composition of the metal or alloys used in the construction of the vessels

3 Ninety-four to ninety-six per cent of copper added as copper sulphate to wheat or to milk is excreted in the faeces. The presence of a very slight but constant quantity of copper in the urine indicates poor absorption of the metal

4 Copper that enters food from the vessels due to contamination is metabolized in a manner very similar to that of added copper. There is, however, indication of a slightly higher excretion in urine and increased retention in the body. This point needs further investigation

5 Groups of rats which were kept on a diet of *chapatti* with supplements of milk to which were added 6 mg and 9 mg of copper as copper sulphate appeared less active, with hairs slightly pigmented and showed growth rate lower than those of the controls

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A PRELIMINARY NOTE ON THE PHARMACOLOGICAL ACTION OF THE ALKALOIDS OF *RAUWOLFIA* *SERPENTINA*

BY

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CHOPRA, GUPTA and MUKHERJEE (1933) reported on the pharmacological action of an alkaloid isolated from *Rauwolfia serpentina*. This alkaloid was found to correspond with ajmaline, one of the principal alkaloids isolated and described by Siddiqui and Siddiqui (1931). Since the isolation in pure form of the different alkaloidal principles of the plant by Siddiqui and Siddiqui (1932), little work has been done on a quantitative basis on the pharmacological action of the individual alkaloids of *Rauwolfia serpentina*. An alcoholic extract of the root is largely used as a circulatory depressant in cases of hyperpiesis and also as a sedative in conditions of hyperexcitability of the central nervous system. As alcoholic extracts contain all the alkaloids it is not known which of them is most potent in lowering the blood-pressure. The present paper is an attempt to make a comparative quantitative study of the three principal alkaloids, namely, ajmaline, serpentine and serpentinine, as regards their relative toxicities and also their respective potencies in lowering the arterial blood-pressure.

Experimental details—The toxicity of the different alkaloids was tested on white mice weighing between 15 and 20 grammes. The different doses of the alkaloids were contained in 0.4 c.c. saline (0.9 per cent) per 20 g. body-weight mouse and were intraperitoneally injected.

Toxic symptoms—The symptoms varied with the magnitude of the dose and the alkaloid injected. Toxic symptoms appeared within 5 minutes in mice injected

with large doses of ajmaline hydrochloride, and the animals died within 10 minutes of such injections. Signs of motor excitability, stiffness of tail, quickening followed by slowing of the respiratory rate, and mild convulsions were the main symptoms observed in the animals injected with lethal doses of the drug.

In animals injected with graded doses of serpentine, symptoms were similar to those observed in animals injected with ajmaline. Serpentine, however, seemed to be a more powerful convulsant than ajmaline.

Neither ajmaline nor serpentine appeared to have any significant sedative effect either in the lethal or sublethal doses while, on the other hand, they seemed to be stimulants of the central nervous system, especially the motor cortex. In mice injected with sublethal doses of serpentinine, prolonged drowsiness with slow respiratory rate was a characteristic symptom. Death was delayed in mice injected with lethal doses of serpentinine. In all cases death was primarily due to failure of respiration, the heart continued beating for some time after the cessation of respiration. The results are given in Table I —

TABLE I

Showing the toxicity of the different alkaloids and crude extracts of Rauwolfia serpentina in white mice by intraperitoneal injections

Dosage mg /20 g mouse	Ajmaline	Serpentine	Serpentinine	Group I (containing the crude extract and ajmaline)	Group II (containing the crude extract, serpentine and serpentinine)
1 00	0/5*				
2 00	0/9	3/3	0/3		
2 12	3/5				
2 25	5/6				
2 50	4/5		3/3	0/3	
3 00	5/5		3/3		
3 50	5/5			0/5	0/4
4 50		3/3	3/3		
5 70				3/5	
8 00					

* Number of mice died/number of mice injected

In Table I some of the doses injected are geometrically mean in between two doses. The coefficient of therapeutic efficiency, namely, the ratio of the lethal dose to the maximum tolerated dose, is small for the alkaloids.

A large number of mice was not used to construct a dose-mortality curve from which LD_{50} of each of the alkaloids could be determined. The LD_{50} was therefore worked out by using Kärber's (1927) formula. The LD_{50} of ajmaline hydrochloride as found by Kärber's method is 2.59, that is, when a dose of 2.59 mg of the drug per 20 g body-weight mouse is intraperitoneally injected it will kill about 50 per cent of the total mice injected. This toxicity figure compares favourably with that reported by Chopra *et al* (*loc cit*) for the same alkaloid.

If the relative mortality figures in Table I are compared it seems that the median lethal dose for serpentinine is not far from that for ajmaline. Dose for dose serpentine appears to be very much more toxic than the other two. If the figures representing the absolute lethal doses are compared, ajmaline appears to be the least toxic followed in order by serpentinine and serpentine.

Groups I and II represent crude extracts containing all the alkaloids and other principles of the plant. Group I contained ajmaline and group II serpentinine and serpentine. It is interesting to note that mortality figures due to these crude extracts are different from those due to the pure alkaloids. It therefore appears that the toxicity of these alkaloids are very much reduced when in combination with other ingredients in the crude extracts. These toxicity-reducing substances have not been precisely found out. The drug is very much used in the form of alcoholic extracts and it seems, therefore, that it is safer to administer it in this form rather than giving the proximate principles individually in which case the toxicity is comparatively higher.

Effects of the different alkaloids on the blood-pressure of decerebrate and spinal cats—Experiments were devised to ascertain the sites of action of these alkaloids so as to group them as central and peripheral circulatory depressants. Decerebrate and spinal cats were used to study their effects on the vasomotor centre in the medulla and the peripheral circulatory apparatuses, namely, the heart and the blood vessels.

In all our experiments a quantitative comparison of the effects of the alkaloids on the blood-pressure was originally aimed at but later it was found that their actions qualitatively differed. The results are given in Table II.

In a few experiments it was observed that some of the alkaloids did not show much depressor effects when the arterial-pressure was low. In some spinal cats where the arterial-pressure was considerably low, a slow continuous intravenous infusion of adrenaline (about 10% per c.c.) was therefore started to raise the pressure. When the pressure rose, ajmaline induced a marked fall in spite of the infusion of adrenaline. Under these conditions the effects due to the other alkaloids were not significant.

It is seen from Table II that serpentine reduced the arterial-pressure of all experimental animals, while the actions due to the other two alkaloids varied

according to the preparation used Ajmaline and serpentinine both raised the blood-pressure, the action being more marked with ajmaline, in the decerebrate preparations. In the spinal preparations where the medullary centres were destroyed, these alkaloids produced fall of pressure. It seems that in decerebrate cats with medulla intact, the stimulation of the vasomotor centre masked the depressant effects on the heart and the blood vessels, the resultant effect being a rise of arterial-pressure. In one spinal preparation, however, serpentinine raised the pressure.

TABLE II

Showing the effects of the individual alkaloids on the blood-pressure of decerebrate and spinal animals -

Alkaloids	Dose, mg	Preparations	BLOOD PRESSURE	
			Rise mm Hg	Fall mm Hg
Ajmaline	2	Decerebrate	23	
	5	"	47	
	10	Spinal		22
	10	"		6
Serpentinine	2	Decerebrate	16	
	5	"	25	
	10	Spinal		13
	10	"	22	
Serpentine	2	Decerebrate		23
	5	"		23
	10	Spinal		40
	10	"		21
Group I	2.5	Decerebrate		46
" II	2.5	"		46

DISCUSSION

The effects of the individual alkaloids on the blood-pressure of animals have not been reported by earlier workers. Sen and Bose (1931) studied the actions of

the crude drug Chopra *et al* (*loc cit*), using ajmaline in spinal preparations, reported a fall of blood-pressure which result is concordant with that found by us. If they had used decerebrate preparations results would have been different. This blood-pressure raising property of ajmaline is largely due to its intense visomotor stimulation.

Serpentine appears to have the most depressor effects. It might be due to the preponderance of serpentine that alcoholic liquid extracts of the root always produce a fall of arterial-pressure though it is also quite probable that such effects are due to the presence of other depressor principles not yet definitely isolated from the root. It is interesting to note that group I containing a mixture of ajmaline and other substances, produced marked fall of blood-pressure in decerebrate preparations (Table II). Group II, though it contained serpentinine and the extract, nevertheless produced a fall of blood-pressure due to the presence of the more powerful serpentine.

The drug is extensively used of late for its sedative action in cases of insanity with violent maniacal symptoms. Neither ajmaline nor serpentine seemed to have any hypnotic effects. Drowsiness was, however, noticed in mice injected with serpentinine and such effect was better marked when the animals were injected with liquid extracts of the root containing mixture of the alkaloids besides other ingredients. Repeated or big doses of ajmaline were found to precipitate convulsions with retching in decerebrate animals. This exciting central action of ajmaline was never observed when crude extracts of the root were used. It seems, therefore, at present that crude extracts or powdered roots are clinically more effective as the undesirable central stimulant action due to some of the alkaloids is masked by the presence of other unknown principles having more sedative and powerful depressant action on the blood-pressure.

SUMMARY

1 The toxicity of the three alkaloids, namely, ajmaline, serpentine and serpentinine, and their toxic symptoms have been studied on white mice by intraperitoneal injections. Serpentine is the most toxic of the three alkaloids. Ajmaline and serpentinine are about equally toxic.

2 Crude extracts of *R. serpentina* produced fall in arterial-pressure which seemed to be peripheral in origin.

3 Ajmaline raised the blood-pressure in decerebrate animals but lowered the pressure in spinal preparations. This fall in pressure was more marked when the pressure was previously raised and maintained at a higher level by slow continuous intravenous injection of adrenaline.

4 Serpentine produced fall in arterial-pressure, both in the decerebrate and in the spinal preparations.

5 The effects of serpentinine closely followed those of ajmaline on the blood-pressure of experimental animals.

6 Neither ajmaline nor serpentine had any narcotic or sleep-producing effects in mice. Serpentinine possessed such effects. Sedative effects were, however, found with the crude extracts.

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PHARMACOLOGICAL ACTION OF THE ESSENTIAL OIL OF *CURCUMA LONGA*

BY

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INTRODUCTION

Of the two species of *Curcuma* growing in India, namely *Curcuma longa* and *C. aromatica*, the former or the real turmeric is extensively cultivated in this country. The rhizome of this plant is popularly known as 'haldi' and is used both as a condiment and a dye. *C. aromatica* Salisb., also known as wild *Curcuma* (Sanskrit Vanaharidra, Hindi Jangli haldi, Bengali Banhalud, Behari Banhaldi, Malayam Kasturimanjal), grows wild in some parts of Mysore State and is probably indigenous to various other parts of India. The plant as a whole contains the colouring matter, the underground rhizome containing the greater part of it.

Turmeric in the indigenous medicine—From times immemorial turmeric has been used by the Ayurvedic and the Unani schools of medicine in India as a stomachic, tonic and a blood purifier. It has its use also as an antiperiodic alterative. Household turmeric mixed with warm milk is said to be beneficial in common cold. The fresh juice from the rhizome, a paste prepared from it or the decoction of the plant, is often employed against leprosy, snake-bite, vomiting of pregnancy and affections of the liver. Murray advocates its use in troublesome diarrhoeas in atonic subjects. Baden Powell found it to be effective in intermittent fevers and dropsy. The fresh juice from the rhizome is believed to be antiparasitic

for many skin affections. Externally it is used for indolent ulcers and a paste made from the powdered rhizome along with caustic lime forms a soothing remedy for inflamed and angry joints. A decoction made from the rhizome is said to relieve pain of purulent ophthalmia. It is still a common practice in India to use a piece of cloth soaked in turmeric solution for wiping away discharges in acute conjunctivitis and ophthalmia. Finely powdered turmeric mixed with alum forms a common household remedy for otorrhœa. A thick watery paste of 'haldi' is used on many auspicious occasions amongst the Hindus in this country. The smoke produced by sprinkling powdered turmeric over glowing charcoal is said to relieve pain due to scorpion bite.

Chemical considerations—The chemical formula of *Curcumin*, the yellow colouring matter of turmeric, is either $C_{10}H_{10}O_3$ or $C_{16}H_{16}O_4$. It melts at $172^{\circ}C$, forms red-brown salts with alkalis, is converted by boric or sulphuric acid into rosocyanine, by reduction with zinc dust into an oily body, by oxidation into oxalic or terephthalic acid and by fusion with potash into protocatechuic acid. Oil of turmeric is distilled from the dried rhizomes of *Curcuma longa*. Fresh rhizomes yield 0.24 per cent of a yellow coloured oil which is soluble in 75 per cent alcohol. Turmeric oil contains *phellandrene*. Oil extracted from Bengal turmeric with petroleum ether when fractionally distilled under reduced pressure gave the following fractions: (1) boiling below $193^{\circ}C$, (2) at $193^{\circ}C$ to $198^{\circ}C$, (3) viscid semi-solid residue. The middle fraction, after purification, seemed to contain an alcohol, 'turmerol', of the composition $C_{19}H_{28}O$ described as a pleasant aromatic smelling oil with a density of 0.901 at $170^{\circ}C$.

PHARMACOLOGICAL ACTION OF *Curcuma* OIL

The Director of the Indian Institute of Science, Bangalore, kindly sent us a sample of the yellow essential oil isolated from *Curcuma longa*. We have attempted to work out the pharmacological actions of the oil, the results of our experiments are given below—

Action on Paramecium caudatum—The effects of *Curcuma* oil in different concentrations were studied on *P. caudatum* kept on a glass-slide with six small chambers. The addition of the oil in concentrations varying from 1 in 2,000 to 1 in 5,000 resulted in paralysis and subsequent death of the organisms, complete disintegration with loss of shape was a marked feature. With weaker dilutions of the oil, such as 1 in 10,000 to 1 in 30,000, the ciliates at first became active but were later sluggish and ultimately died within 10 to 30 minutes. The organism did not undergo any change with higher dilutions of the oil, such as 1 in 50,000 to 1 in 100,000.

Action on the bacteria—The bactericidal properties of the oil were tested on *Staphylococcus albus* and *aureus*, and *B. typhosus*.

The growth of cultures of *Staphylococcus aureus* and *albus* was inhibited in concentrations up to 1 in 5,000. The growth of cultures of *B. typhosus* was not inhibited even in a concentration of 1 in 1,000.

Local action—The essential oil has a strong aromatic odour, and applied locally it has a slightly irritant action on the unbroken skin. Applied to the mucous membranes it produces well-marked vasodilatation. A one per cent solution of the oil when instilled into the eyes of a rabbit produces slight redness of the conjunctiva but does not produce any local anaesthesia. One or 2 per cent solution of the oil if injected deep into thigh muscles of a cat does not produce unpleasant symptoms or appreciable congestion, oedema or necrosis at the site of the injection.

Action on the gastro-intestinal tract (a) *Carminative effect*—Taken by mouth, the solution of the oil has warm aromatic taste and promotes the flow of saliva. Taken internally in doses of 5 to 10 minims suspended in water the oil gives rise to a feeling of warmth and sense of comfort in the stomach. It, therefore, seems to act as an appetizer, stomachic and tonic. Large doses, such as 2 c c to 4 c c of the concentrated solution of the oil, give rise to sharp pain and discomfort in the epigastrium and induce salivation, retching and vomiting.

(b) *Effects on the gastric secretion*—Free and total acid contents of the gastric juice were determined on the same subject on two different days, observations being made on patients in the Carmichael Hospital for Tropical Diseases during fasting. A Ryle's tube was introduced into the stomach and 15-minute samples were aspirated and collected for examinations on two different days. On the first day the samples were collected after administration of 50 c c of 7 per cent ethyl alcohol alone and on another day, after 50 c c of 7 per cent ethyl alcohol administered after 10 c c of 1 per cent *Curcuma* oil. The contents were withdrawn every 15 minutes for 2½ hours and the total and free acids were determined.

The results obtained after fractional test meal on different individuals show that the administration of *Curcuma* oil is followed by a marked diminution of secretion of the acids in the stomach.

(c) *Action on the small intestine*—The movements of the small intestine were studied in chloralosed cats with Jackson's enterograph. The essential oil in 1 c c to 2 c c doses of a 1 per cent solution at first produced slight increase of the tone followed by subsequent relaxation of peristaltic movements. The injections of the essential oil to the isolated pieces of kitten's intestine in the Dale's bath produced similar but less marked effects.

Action on the cardiovascular system (i) *On the blood-pressure*—The oil injected in different doses intravenously into cats under urethane and chloralose anaesthesia always produced a fall in blood-pressure. With smaller doses, such as 1 c c or 2 c c of a 1 per cent solution of the oil, the fall in blood-pressure was abrupt but recovery was also rapid. This fall in pressure was, however, marked and maintained with larger doses, such as 2 c c to 4 c c, but the pressure in all experiments returned to normal.

(ii) *Action on the blood vessels*—The fall in arterial blood-pressure was always followed by a decrease in the volume of the intestine and the spleen but with a rise of the limb volume. The re-distribution of the blood, therefore, appears to be directed towards the peripheral blood vessels which show dilatation.

(iii) *Action on the heart*—The myocardiograph experiments in the anaesthetized cats showed slight stimulation of the auricles with dilatation of the ventricles. The injections of larger doses such as 2 c c to 5 c c, however, resulted in diminution of the amplitude of contractions of both the auricles and ventricles.

Action on the respiratory system—The effects of the essential oil were studied on the respiration of cats under urethane anaesthesia. An increase in both the rate and the amplitude of the respiratory movements was obtained with smaller doses, such as 1 c c or 2 c c of a 1 per cent solution of the oil.

SUMMARY AND CONCLUSIONS

1 The essential oil of *Curcuma longa* appears to have feeble antiseptic properties.

2 The secretion of acid of the gastric juice is inhibited, it acts as an antacid.

3 It seems to share the carminative property of other essential oils. In large doses it appears to act as an anti-spasmodic inhibiting the excessive peristaltic movements of the intestine.

4 The effects of the oil on the cardiovascular and the respiratory system are not marked and therefore not of much importance from therapeutic point of view.

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LECITHIN AND HÆMOLYSIS

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LECITHIN is one of those substances the functions and properties of which have not been fully assessed. The findings of different investigators on many points have been contradictory. This is principally due to the difficulty of obtaining it in a pure condition and to its extremely labile nature. It undergoes rapid changes in the presence of air and light. Many of the properties of lecithin are closely associated with the presence of highly unsaturated fatty acids in the molecule. These acids confer upon lecithin the property of absorbing oxygen with great rapidity so that a freshly prepared pure sample of lecithin which is clear and wax-like in appearance may change on exposure to air into a brown or dark-brown mass within a very short time, differing entirely from the unoxidized specimen with regard to solubility and other properties. Much of the confusion which prevails in the literature respecting the behaviour of lecithin is due to the failure to take note of the facts mentioned above.

The association of lecithin with hæmolytic phenomenon dates back to 1903 when Kyes discovered its remarkable property of accelerating venom hæmolysis. It is well known that the erythrocytes of various animals differ in their susceptibility to venom hæmolysis to a remarkable extent. For instance, those of the dog, the guinea-pig and the cat are most susceptible to the process, those of the ox, the goat and the sheep are entirely refractory, i.e. they are not lysed by any of the venoms, whereas those of man, rat, rabbit, pig, horse, etc. occupy an

intermediate position and have varying degrees of susceptibility. Venoms obtained from different species of snakes also vary considerably as regards their hæmolytic activity—those of the *Colubridæ venenosæ* class are more or less hæmolytic, whilst those belonging to the *Viperidæ* species are either non-hæmolytic or hæmolytic only to a very limited extent.

In the presence of a trace of lecithin, however, all the different varieties of venoms, hæmolytic or non-hæmolytic, hæmolyse the erythrocytes obtained from all the different species of animals, susceptible or refractory, very readily. It is therefore evident that the role of lecithin in connection with venom hæmolysis is very significant.

The acceleration of venom hæmolysis in the presence of lecithin is believed to be due to a very active hæmolysin formed by the enzymatic action of lecithinase present in the venoms. Roy (1938) made a comparative study of the lecithinase activity of cobra and Russell's viper venom and subsequently of echis venom and found that all three contained active enzymes capable of splitting up lecithin, the Russell's viper venom being the most active in this respect.

Without entering into any discussion as to the mechanism of lecithin-venom hæmolysis for the present (to be discussed in detail elsewhere) the fact that lecithin activates all kinds of venom hæmolysis remains undisputed.

This remarkable property of lecithin led subsequent workers to study its action on other substances as well, with the result that statements like the following appeared from time to time describing its action on substances of varied nature both organic and inorganic. Kritschewsky and Muratowa (1922) stated that quinine, like cobra venom, was activated by lecithin. Pascucci (1905) observed that the agglutinating capacity of ricin was converted into hæmolytic activity after the addition of lecithin. Arrhenius (1908) observed that lecithin activated the hæmolysis caused by acetates and boric acid.

Friede (1924) studied the action of lecithin on a number of substances of varied nature selected at random. He found that there was a group of substances which were hæmolytic themselves and that the addition of lecithin served to accelerate such hæmolysis. Mono- and dihydrochlorides of quinine, quinine sulphate, optochin, gallic acid, anilin (probably the hydrochloride), arsenic acid, carbolic acid, acetates, boric acid and silicic acid, according to him, fall under this head. Another group of substances was found by him to be non-hæmolytic, but they hæmolysed in combination with lecithin, e.g. caffeine, cocaine, strychnine, chloral hydrate and potassium bromide.

A third group of substances was strongly hæmolytic but lecithin was found to be without any effect on their action, e.g. saponin, tetanotoxin, sodium taurocholate, salvarsan and mercuric chloride.

The result of Friede's observations is well summarized by the following statements of his own: 'So we see similar to animal poisons, there are many chemically defined substances, as well as organic and mineral poisons and medicinal

substances, which have the capacity to hæmolyse in combination with lecithin, even in those cases where the substances themselves in high concentrations do not hæmolyse' (The English translation is ours)

The activation of venom hæmolysis by lecithin forms a distinct class by itself because these venoms in spite of their very complex nature have one thing in common, in that they all contain an enzyme capable of splitting up lecithin with the formation of strong hæmolytic substances. It appeared to us to be very peculiar that lecithin should have similar action on a number of other substances of widely divergent nature for which no reasonable explanation could be given.

Accordingly, the action of lecithin on some of those substances was studied. For this investigation Merck's lecithin (ex ovo, light brown) which was repeatedly washed with hot acetone to get rid of free fatty acids was employed. A 1.0 per cent emulsion of lecithin was prepared from the purified specimen from which more dilute solutions were prepared when required. This stock solution would keep for several weeks when stored in a glass-stoppered container inside an ice-chest.

The salts of quinine first engaged our attention because of their clinical interest. Krichewsky and Muratowa (*loc cit*) supposed that hæmoglobinuria in malaria was the result of the ingestion of quinine. When the concentration of quinine in the blood is sufficiently high, it is said to hæmolyse in combination with lecithin present in the serum. The salts of quinine, however, are not administered in such doses as can produce a concentration in the blood approaching that which can produce hæmolysis. Moreover, cases of hæmoglobinuria do occur in malaria, without administration of quinine. It is also known that quinine in whatever form administered appears in the circulating blood in the form of the base. The solubility of quinine alkaloid is stated by Barratt and Yorke (1909) to be about 1 in 1,600 in water. According to Christophers (1929) with a saturated solution of quinine in salt which gave a pH of 9.4, complete hæmolysis was obtained. At half this concentration, giving a pH of 9.2, no hæmolysis was obtained in three hours or overnight. It is therefore highly improbable that the quinine alkaloid should ever attain such concentration in the blood as to cause hæmolysis even assuming that lecithin has an accelerating action upon it.

Quinine bihydrochloride —According to Friede (*loc cit*) this salt in a dilution of 1 in 100 produces complete hæmolysis, almost complete hæmolysis in 1 in 150 but in a dilution of 1 in 200 and upwards it ceases to be hæmolytic. In the presence of lecithin 1 in 10,000, however, complete hæmolysis was obtained even in a dilution of 1 in 200.

With a Howard's preparation of quinine bihydrochloride the following results were obtained. The pH in each case was determined electrometrically with quinhydrone electrode on solutions which contained the same concentration of quinine hydrochloride but in place of the washed r b c an equal volume of normal saline was used.

It appears from Table I that lecithin has no accelerating action on the hæmolysis caused by quinine bihydrochloride and that hæmolysis is due to the acidity of the system. In this connection it will be pertinent to recall the following remarks of Christophers (*loc cit*) —

‘This salt conforms in every way with the action of an acid salt, i.e. its limit of hæmolytic action is at a pH point of about 4.6 and being highly dissociated and hydrolysed, it gives almost the same result as free HCl. The salt behaves as though it had but one available molecule of HCl.’

TABLE I

Number	Sheep's rbc 3 per cent, cc	Quinine 2HCl 1 in 500, cc	Normal saline, cc	Lecithin emulsion 1 in 1,000	Immediate	$\frac{1}{2}$ hour	1 hour	2 hours	24 hours	pH with cit blood
1	0.3	0.5	0.2	—	+++	+++++	+++++	+++++	+++++	4.04
2	0.3	0.5	0.1	0.1	+++	+++++	+++++	+++++	+++++	3.91
3	0.3	0.2	0.5	—	—	—	—	—	—	4.40
4	0.3	0.2	0.4	0.1	—	—	—	—	—	4.18
5	0.3	0.05	0.65	—	—	—	—	—	—	5.66
6	0.3	0.05	0.55	0.1	—	—	—	—	—	5.31
7	0.3	—	0.7	—	—	—	—	—	—	6.53
8	0.3	—	0.6	0.1	—	—	—	—	—	6.50

+++++
±
—

Complete hæmolysis
Doubtful hæmolysis
No hæmolysis

Quinine monochloride — According to Friede this salt produced almost complete hæmolysis at a dilution of 1 in 100, and with 1 in 150 solution no hæmolysis took place, but with lecithin (1 in 10,000) a 1 in 500 dilution of the salt will produce complete hæmolysis.

Table II shows the results of our experiments with quinine monohydrochloride (Howard) —

TABLE II

Number	Sheep's rbc 3 percent, cc	Quinine HCl 1 in 100, cc	Normal saline, cc	Lecithin 1 in 1,000, cc	$\frac{1}{2}$ hour	1 hour	2 hours	24 hours	pH without blood
1	0.3	0.5	0.2	—	—	—	+++	+++++	6.80
2	0.3	0.5	0.1	0.1	—	—	+++	+++++	6.08
3	0.3	0.2	0.5	—	—	—	—	—	7.07
4	0.3	0.2	0.4	0.1	—	—	—	—	6.01
5	0.3	—	0.7	—	—	—	—	—	6.02
6	0.3	—	0.6	0.1	—	—	—	—	7.45

See Note under Table I

It would appear that lecithin has no activating action on this salt also. It is indeed very difficult to connect the hæmolytic action of this salt with its acidity unless it is assumed that the addition of red cells causes an appreciable increase of the pH due to increased dissociation.

Christophers (*loc cit*) considers this salt to be like boric acid which is hæmolytic with a pH far below that of the unbuffered acids. According to him the peculiar results given by quinine monohydrochloride are due to special conditions prevailing in regard to dissociation and not to 'special' hæmolytic properties proper to quinine as such. Very small changes might appear to enhance the hæmolytic effect when all that had really happened was some increase in dissociation.

Quinine sulphate—This salt is said to produce complete hæmolysis in dilutions up to 1 in 6,000. It is not hæmolytic in a dilution of 1 in 8,000 and upwards.

(Friede, *loc cit*) In combination with 1 in 10,000 lecithin, however, 1 in 10,000 dilution of this salt produces complete hæmolysis. With 1 in 500 solution of quinine sulphate (Howard) the following results were obtained —

TABLE III

Number	Sheep's rbc 3 per cent, cc	Quinine sulphate 1 in 500, cc	Normal saline, cc	Lecithin 1 in 1,000, cc		1 hour	2 hours	21 hours	pH without blood
1	0.3	0.5	0.2	—	—	—	—	±	6.84
2	0.3	0.5	0.1	0.1	—	—	—	—	7.03
3	0.3	0.2	0.5	—	—	—	—	+	6.96
4	0.3	0.2	0.4	0.1	—	—	—	—	7.02
5	0.3	0.05	0.65	—	—	—	—	+	7.29
6	0.3	0.05	0.55	0.1	—	—	—	—	6.94
7	0.3	—	0.7	—	—	—	—	+	6.85
8	0.3	—	0.6	0.1	—	—	—	—	6.98

See Note under Table I

The above results show that quinine sulphate is not hæmolytic even in a dilution of 1 in 1,000 and that lecithin has no accelerating action upon such hæmolysis. It is probable that Friede had used a specimen of this salt which contained appreciable amounts of free sulphuric acid. In this connection the following remarks of Christophers are worth quoting 'soluble 1 in 800 of water. This salt is not hæmolytic in saturated solution in salt (0.0014 mol per litre). Being a bivalent salt it is likely to be less dissociated in solution than the other salts mentioned and, being relatively insoluble, it does not give the necessary concentration of acid for hæmolysis. In saturated solution in 0.85 salt the pH is about 5.8'

Optochin — This compound is said to produce almost complete hæmolysis in a dilution of 1 in 250 and no hæmolysis in 1 in 300 solution, but with this latter

dilution complete hæmolysis is said to take place with lecithin (1 in 50,000) The hydrochloride used for our experiments was prepared from the base ethyl hydrocupreine (Merck's) by the addition of the calculated amount of hydrochloric acid and making up to the concentration required Sodium chloride was then added to make it 0.85 per cent The concentrations are expressed in terms of the base

TABLE IV

Optochin (HCl) 1/200

Number	Rbc, cc	Optochin, cc	Normal saline, cc	Lecithin 1 in 1,000, cc	½ hour	1 hour	2 hours	21 hours	pH colorimetric
1	0.3	0.5	0.2	—	+++++	+++++	+++++	+++++	4.7
2	0.3	0.5	0.1	0.1	+++++	+++++	+++++	+++++	—
3	0.3	0.2	0.5	—	—	—	—	++++	4.9
4	0.3	0.2	0.4	0.1	—	—	—	++++	—
5	0.3	0.1	0.6	—	—	—	—	—	5.1
6	0.3	0.1	0.5	0.1	—	—	—	—	—
7	0.3	0.05	0.65	—	—	—	—	—	6.0
8	0.3	0.05	0.55	0.1	—	—	—	—	—

See Note under Table I

It would appear from Table IV that, with and without lecithin the various concentrations of optochin hæmolysed at the same rate and that the hæmolytic action is due to the acidity of the solution This is borne out by the fact that in the first two tubes where hæmolysis takes place comparatively quickly the hæmolysed solutions have a greenish yellow colour characteristic of acid hæmolysis The same remark holds good with respect to aspirin also, which even in a dilution of 1 in 4,000 has a pH of about 4.0

It is said (Pascucci, *loc cit*) that ricin alone produces agglutination of r b c but in combination with lecithin, hæmolysis takes place. Our results show that with or without lecithin ricin produces only agglutination of the red blood corpuscles and in no case hæmolysis is produced.

The action of lecithin on bacterial hæmolysins, e.g. vibrio (El Tor) hæmolysin and streptococcal hæmolysin, was also studied. Vibrios were grown in 1 per cent peptone solution and *Streptococcus hæmolyticus* in serum broth. Eighteen hours old cultures were used in both the cases without centrifugation. It would appear from Table V that lecithin has no appreciable action on vibrio hæmolysins —

TABLE V

Number	R b c, c c	Culture fluid, c c	Normal saline, c c	Lecithin 1 in 100, c c	$\frac{1}{2}$ hour
1	0.3	0.1	0.6	—	+++++
2	0.3	0.1	0.1	0.5	+++++
3	0.3	0.1	0.4	0.2	+++++
4	0.3	0.1	0.5	0.1	+++++
5	0.3	0.1	0.55	0.05	+++++
6	0.3	—	0.7	—	—

See Note under Table I

Lecithin, however, has a marked inhibitory action on streptococcal hæmolysin as Table VI will show. These results therefore confirm the findings of Gordon and Stansfield (1929) —

TABLE VI

Number	R b c, c c	Culture fluid, c c	Normal saline, c c	Lecithin 10 per cent, c c	$\frac{1}{2}$ hour	1 hour	2 hours	21 hours
1	0.3	0.1	0.6	—	+++++	+++++	+++++	+++++
2	0.3	0.1	0.55	0.05	—	—	—	—
3	0.3	0.1	0.5	0.1	—	—	—	—
4	0.3	0.1	0.3	0.3	—	—	—	—
5	0.3	0.1	0.1	0.5	—	—	—	—
6	0.3	—	0.7	—	—	—	—	—

See Note under Table I

It appears therefore that excepting in the case of snake venoms and some other animal poisons, which contain active enzymes (lecithinase), purified lecithin does not accelerate hæmolysis. The acceleration observed by some previous workers in the large majority of cases was due either to the fatty acids present in the impure specimen of lecithin or to some other extrinsic factor.

SUMMARY AND CONCLUSIONS

- 1 The action of lecithin on hæmolytic activity of salts of quinine and optochin as well as on some bacterial hæmolysins was studied.
- 2 The hæmolytic action of the salts of quinine and optochin seems to depend upon their acidity as these salts are easily hydrolysed.
- 3 A purified sample of lecithin had no action on the hæmolysis caused by any of these salts.
- 4 The acceleration observed by previous workers was evidently due to the free fatty acids present in impure specimens of lecithin which they used.
- 5 Purified lecithin had no appreciable action upon vibrio (El Tor) hæmolysin but it has a marked inhibitory action on *streptococcal* hæmolysin.

ACKNOWLEDGMENT

We are thankful to Mr P Mukherjee, B Sc, for his valuable help in this connection.

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FURTHER INVESTIGATIONS ON THE TRANSMISSION OF KALA-AZAR

Part IV

THE DURATION OF LIFE AND OTHER OBSERVATIONS ON 'BLOCKED' FLIES

BY

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[Received for publication, June 5 1941]

THE detection of sandflies *P argentipes*, so heavily infected with flagellates that the passage to the midgut was completely obstructed resulting in the inability of such flies to obtain a meal of blood was first made in August 1939, and certain observations regarding the phenomenon were reported in Part II of this series (Smith, Halder and Ahmed, 1940)

Feeding experiments in the 1940 programme were begun on the 6th of May and the first 'blocked' fly of the season was found on the 25th, others were detected in every month till the end of October. The flies used in the experiments were kept in an unregulated incubator in the same manner as the year before, and during the period in question the reading of a wet-bulb thermometer placed inside the incubator varied between a maximum of 87°F and a minimum of 79°F. It is most probable therefore that last year 'blocking' was not detected in the earlier months because we were unfamiliar with the phenomenon, and not that it occurs only at a particular season of the year.

The number of flies suspected to be 'blocked' each month, the results of dissection and the order of their dying is given below in the Table —

TABLE

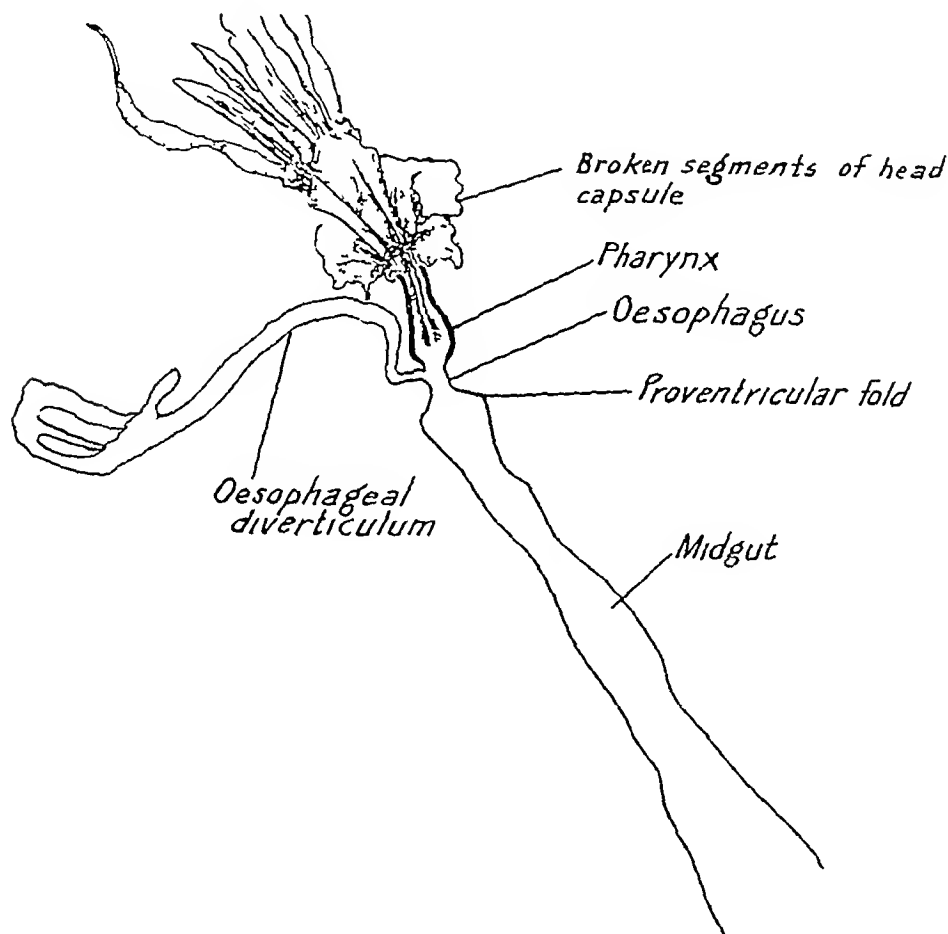
Month	Total	Positive	Negative	Unknown	ORDER OF DYING OF POSITIVE FLIES						
					After the _____ day —						
					1st	2nd	3rd	4th	5th	7th	Total
May	28	18	4	6	10	2	3	1			16
June	21	16	2	3	11	2	2	1			16
July	30	23	1	6	11	2	5	3			21
August	13	9	2	2	2	1	3	1	1	1	9
September	6	6	0	0	2	4					6
October	7	5	0	2	4	1					5
TOTALS	105	77	9	19	39	13	13	6	1	1	73*

* Excludes four flies prepared for section cutting

The diminution in number of 'blocked' flies during and after September was due to the fact that from July onwards parallel feeding experiments with two series of sandflies maintained on raisins and repeated blood meals respectively were being conducted. Both series of flies were infected on the same patient in the same cage, and separated into different batches three or four days later, when those that had oviposited and taken a second blood meal were placed in one category and given the third and subsequent feeds on experimental animals, while the remainder constituted the other, to be maintained on raisins till the tenth day before being offered a second blood meal on an experimental animal. Particular care was taken to detect evidence of blocking during the feeding of the flies, but in no instance was a 'blocked' fly found among those given repeated blood meals, the condition was observed exclusively among the raisin-fed series and as reported in the previous communication only among those being offered a second blood meal.

Of the 105 flies suspected to be 'blocked' 77 were infected with flagellates and 9 were not. In 19 no result was possible owing to decomposition of the flies. Excluding 4 flies which were prepared for sectioning the order of dying of the 73 positive ones was as follows: 39 within the first 24 hours, 13 each on the second and third day, 6 on the fourth and one each on the fifth and seventh day. Although the numbers are small, the longevity of 'blocked' flies would seem to have been greatest in August, when one fly survived for seven days. With few exceptions

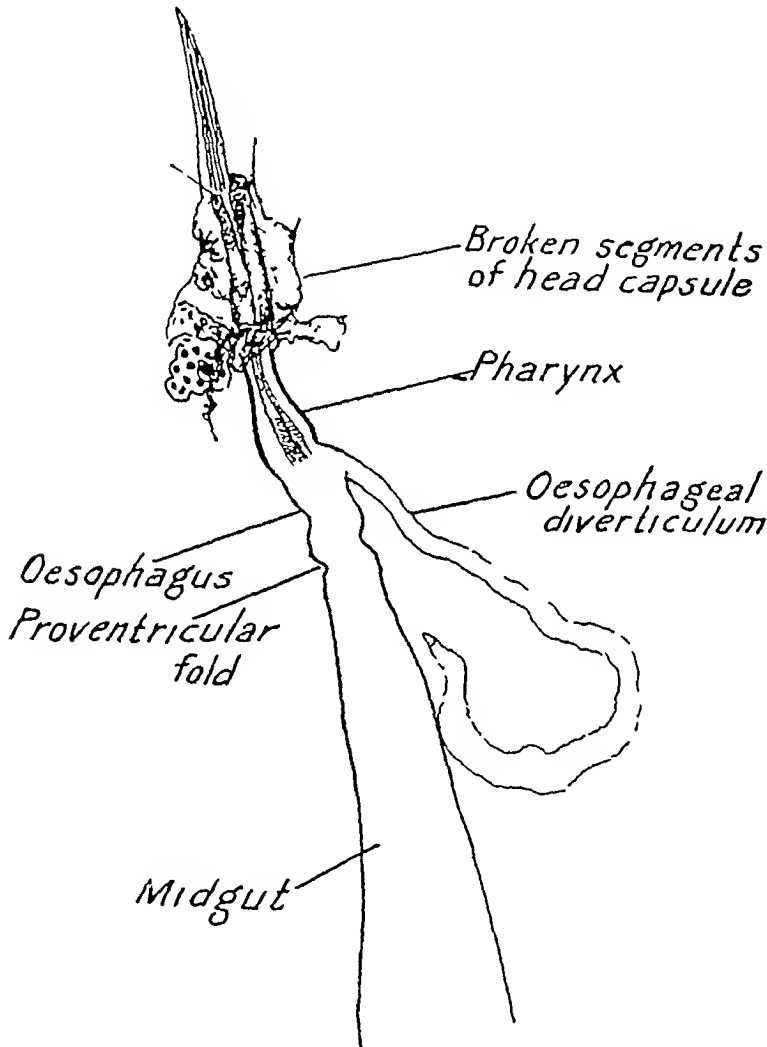
these flies attempted to feed at least once each day when offered opportunities so to do, and more often than not any fly which refused to try was found dead the next morning. Many flies were found to make sustained efforts to obtain a meal of blood two or three times on the same day when given opportunities at intervals of an hour or so, some flies would pierce the skin and not move from that spot till they were apparently weary of trying, others would make attempts at two or three different places for as many minutes at a time before they gave up. The longest period recorded to have been spent by a fly in trying to obtain a meal from one spot was 18 minutes.



TEXT FIGURE 1—Infected fly not 'blocked'

After they were tested each day with the offer of a blood meal the 'blocked' flies were tubed with raisins, from which it is assumed they were able to derive some nourishment, else it is very improbable that the figures for longevity recorded

above would have been obtained. It is possible that a 'blocked' fly, though unable to imbibe sufficient blood to satisfy its hunger, may succeed in procuring small quantities of tissue fluid or plasma which will sustain it for a short time; but till further knowledge of the bionomics of this species is available it would be unwise, we think, to gauge the duration of life of a 'blocked' fly under natural conditions by estimations made in a laboratory.



TEXT FIGURE 2 — 'Blocked' fly

The repeated and persistent efforts made by 'blocked' flies to obtain a meal, would suggest that under natural conditions infections with *L. donovani* are the result not of a single bite from an infected *P. argentipes*, but repeated ones, probably throughout the course of a night on a victim sleeping in a particular part of the room favoured by sandflies, such as a corner away from the prevailing breeze.

If this were so it would be one reason for the slow spread of kala-azar generally, but the number of naturally infected *P. argentipes* found up to date is remarkably small, and quite out of proportion to the incidence of the disease in endemic areas. The paucity of heavily infected flies among such as have been found naturally infected may be due in part to certain discrepancies in the routine adopted up to the present, hitherto both in the collection and dissection of flies from infected dwellings it was the practice to disregard those which showed no evidence of having had a blood meal. 'Blocked' flies have empty midguts and cannot easily be distinguished from newly hatched and unfed females, it is possible therefore that many an infected fly was missed by omitting to examine apparently unfed individuals.

Camera lucida drawings of the anterior halves of the alimentary canals of two flies are presented, one from a 'blocked' fly and the other from an ordinarily infected one which died after a fifth blood meal. The specimens were fixed in Bless solution for three to five minutes and lightly coloured with Leishman's stain to render the outlines more distinct. The distension of the oesophagus of the 'blocked' fly is well seen in contrast to that of the other.

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FURTHER INVESTIGATIONS ON THE TRANSMISSION OF KALA-AZAR

Part V

AN INQUIRY INTO THE RELATION BETWEEN MALARIA AND KALA-AZAR IN A RURAL AREA

BY

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IN his book on 'Kala-azar' and elsewhere Napier (1927) has suggested that kala-azar is the clinical expression, determined by some secondary factor, of an infection that is far more widespread than the disease itself, and that typhoid fever under urban conditions and malaria in rural areas are amongst the secondary factors that determine the morbidity of leishmania infection. In an article on a theory of the aetiology and epidemiology of kala-azar, Napier and Krishnan (1931) present various clinical and other observations in support of this theory and suggest that a dissemination of the infection takes place when, during an attack of either malaria or typhoid, the reaction in the reticulo-endothelial tissues and the mobilization of large mononuclears in the blood results in the transfer of leishmania-laden cells from a quiescent focus of infection in the skin to the visceral endothelial tissues, thereby establishing a general visceral infection.

In their cytological studies of the blood and tissues in kala-azar and associated conditions, Napier, Krishnan and Lal (1933) find further support for the above

theory, and the reason why malaria parasites are so seldom found in the blood of kala-azar patients is stated to be due to the activity of histiocytes in engulfing and destroying parasitized cells, the marked histiocyte proliferation present in kala-azar therefore may be the factor responsible for suppressing the plasmodial infection, but, when co-incident with the cure of kala-azar the histiocytosis disappears, malaria not infrequently supervenes, as is evidenced by typical clinical attacks of the disease often with parasites in the peripheral circulation of patients convalescent after kala-azar

An attempt to obtain experimental support for this theory was made in 1933 (Napier, Smith and Krishnan, 1933) Monkeys of two species were placed under experiment, one *M. mulatta* very susceptible to infections with *P. knowlesi* (the plasmodium used) but resistant to leishmania, and the other *M. irus*, fairly resistant to malaria but susceptible to leishmania. The results were inconclusive, although some evidence was obtained that the less-resistant species was rendered still more susceptible to an attack of leishmaniasis as a result of the plasmodial infection, the result with the other species *M. mulatta* was completely negative

The fact that hæmoglobin is such an essential constituent of media for the growth of leishmania led to an experiment last year to determine whether loading the cells of the reticulo-endothelial system with hæmoglobin, which may be presumed to occur naturally in malaria, would influence an infection with *L. donovani* in an animal. To this end a number of mice were inoculated with a small dose of flagellates, the contents of one infected sandfly each, and a similar number were given in addition, two injections of washed rabbit red blood cells on the days following the inoculation of flagellates. Of 13 animals that survived the period of incubation, three of seven that had cells in addition to flagellates were found infected, and the six controls were all negative. The result is not as decisive as it might be, and whether hæmoglobin acted in the manner visualized above or not, the result we think is not unfavourable to the theory that malaria predisposes to kala-azar

The close correlation in the distribution of malaria and kala-azar in various parts of this district, where localized epidemics of malaria have been followed by considerable increases in the incidence of kala-azar, presented an excellent opportunity to study the connection between the two diseases under natural conditions

The area chosen for investigation was one where an epidemic of malaria began in August 1938, the number of fever cases at that time was not large, but by the autumn of 1939 the epidemic was at its height, and there were reports of many fatalities. A treatment centre opened by the health authorities was visited in November 1939, and 10 samples of blood for aldehyde tests from certain patients with fairly large spleens and 36 blood slides from others were collected for examination. In 30 of the 36 slides malaria parasites were found, but the tests for kala-azar were all completely negative. For various reasons it was not possible to begin the work here reported before March 1940, when with the assistance of the District Health Officer a treatment centre was opened in one of the most heavily

infected villages Arrangements were made for a doctor to visit the centre two days each week and treat all cases of malaria and kala-azar, the junior author (I A) visited the centre once weekly and the senior once a month An endeavour was made to examine a blood smear for malaria from every fresh case attending for treatment before the administration of quinine, to determine in what proportion of patients diagnosed malaria by direct methods, kala-azar would later supervene All fresh cases with enlargement of the spleen two inches or more below the costal margin were also examined for kala-azar by the aldehyde test

Certain drawbacks to the collection of accurate figures regarding the incidence of malaria should here be mentioned the population was an uncontrolled one and the fact that the centre operated only twice each week resulted in a certain number of persons obtaining quinine from other dispensaries or from the bottle of an obliging neighbour on days when the centre was not functioning, others would send a friend or relative for mixture when they were too ill or felt disinclined to walk to the centre themselves These discrepancies are offset to a certain extent by the fact that the amount of quinine in each dose of mixture was small and tended to become smaller still when, not infrequently, local stocks of the drug were low Also we have reason to believe that in the case of small children, more often than not, they were carried to the centre for examination and treatment In view of the fact that the centre was controlled by the district authorities, no patient was refused treatment, and the inclusion of a certain number from villages outside the area of investigation but within that of the epidemic zone, should not it is expected influence the result one way or the other

The previous figures for kala-azar from the villages in question according to the treatment registers of the two closest hospitals are given in Table I —

TABLE I

	1937	1938	1939	1940 (till March)
Tajpur	10	3	33	24
Samastipur	Not available		12	10

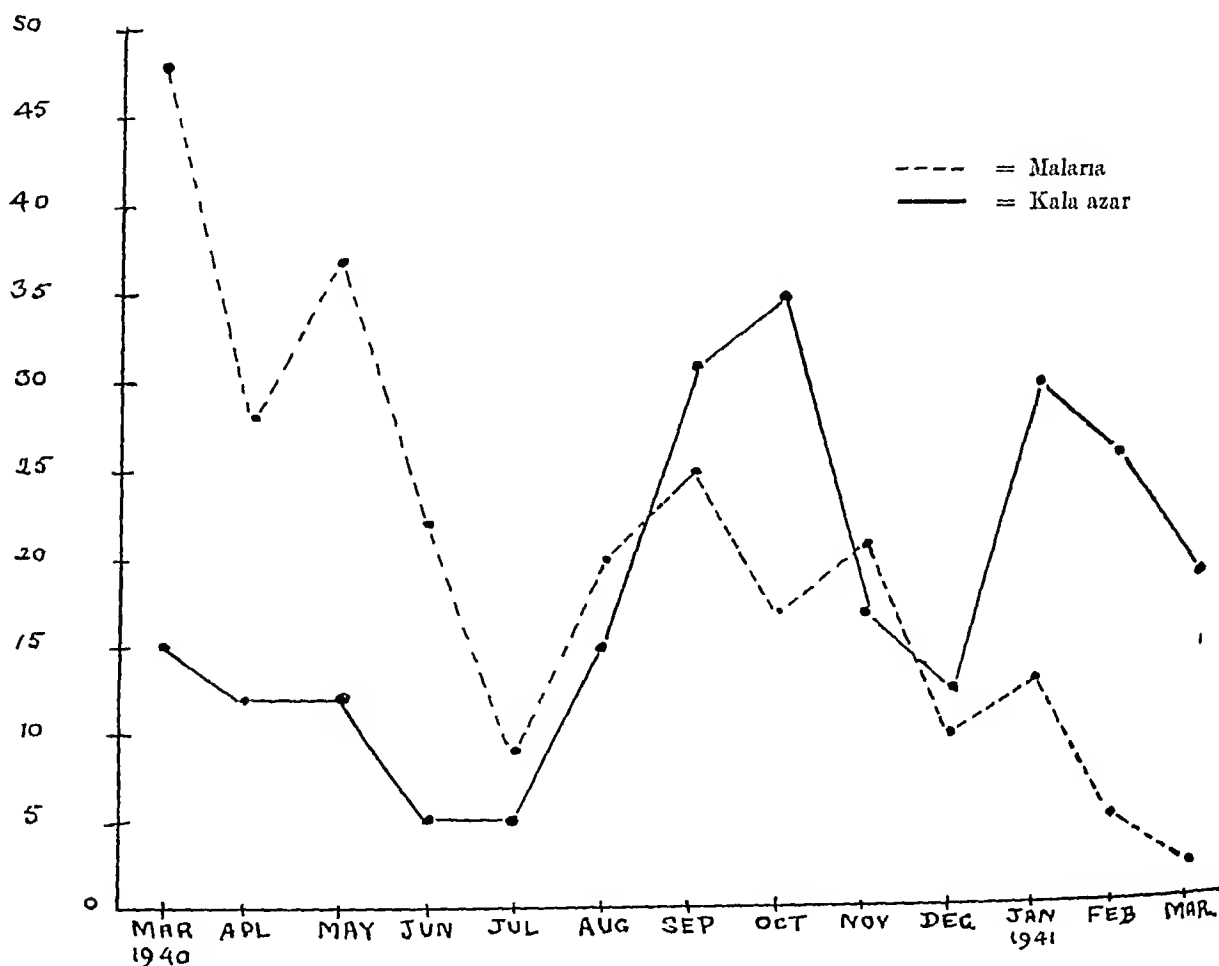
It will be seen that there was an increase in the number of cases attending for treatment in 1939 and the figures up to March would predict a still higher total for 1940 The subsequent history of malaria and kala-azar for the period of

observation may be seen from the Graph showing the number of patients diagnosed each month at the centre

A total of 900 patients were examined once or more, and of these 234 were diagnosed kala-azar and 257 malaria. The total for malaria does not include those

GRAPH

Cases



cases which subsequently developed kala-azar or in which both conditions were found at the same time. Of the 257 cases of malaria there were 110 benign tertian, 118 malignant tertian, 27 mixed, benign and malignant tertian and 2 quartan infections.

The age incidence of these two groups is given in Table II —

TABLE II

Years	Kala azar	Malaria
1 to 5	38	36
6 to 10	107	76
11 to 15	50	49
16 to 20	23	45
21 to 25	6	17
26 to 30	6	16
Over 30	4	18

All but 33 of the kala-azar patients were diagnosed as a result of a positive aldehyde test, and the method of diagnosis and degree of enlargement of the spleen below the costal margin is given in Table III —

TABLE III

Size of spleen	Aldehyde test	Culture	Clinical	L D body	TOTAL.
2 inches and less	54	10	1	0	65
2 to 3 inches	77	13	0	1	91
3 to 4	32	4	1	0	37
4 to 5	29	2	0	0	31
Over 5	9	1	0	0	10

Culture of the blood to arrive at a diagnosis was resorted to in January 1941 on finding a number of suspicious cases which gave negative or incomplete results with the aldehyde test, and some by the urea stibamine method as well. Of 39 cases so examined, flagellates were grown in 30, five were negative and four septic. Among these there were six that had previous histories of malaria, of which three were positive and three negative. Two of the positive cases had had malaria in March and May 1940 and one in September.

An examination of the results shows that among the 234 cases of kala-azar an association with malaria was detected in only 15. In seven instances malaria preceded kala-azar, and in nine, including one of the seven mentioned previously, malaria parasites were found at the same time as a positive aldehyde test was obtained. Details of these cases are given in Table IV —

TABLE IV

Number	Age	Size of spleen, inches	Malaria on	Type of parasite	Aldehyde test, positive on	Size of spleen, inches
1	18	4	6-3-40	B T	24-7-40	4
2	6	1½	22-5-40	B T	2-10-40	3
3	14	3	22-11-39	B T and M T		
			13-4-40	M T	13-4-40	3
4	6	1½	25-9-40	B T	19-3-41	2½
5	6	½	4-9-40	B T	5-3-41	4½ (culture)
6	5	2½	2-3-40	M T	5-3-41	2½ „
7	7	½	1-5-40	B T	12-2-41	1 ,

Aldehyde positive cases showing malaria parasites

1	12	2	22-5-40	B T
2	20	5	13-4-40	M T
3	18	6	25-9-40	M T
4	11	3½	1-5-40	B T
5	6	1½	15-5-40	B T
6	7	2½	11-9-40	M T
7	12	2½	18-9-40	B T
8	6	3	9-3-40	B T
9	Case No 3 in above list			M T

Thirty-two patients were examined once or more for malaria with negative results before a diagnosis of kala-azar was made, but in the remaining 195 instances kala-azar was found already established at the first visit of the patient to the centre. Such a finding would seem to detract largely from the value of the investigation which had for its object the examination of as many cases as possible for malaria before the onset of kala-azar, this finding may be ascribed

partly to the nature of the disease itself, which can often be slow and insidious in its development, and partly also to the mentality of the people who would not attend the centre for treatment till either a convenient opportunity arose or the persistence and increasing severity of symptoms compelled them to seek relief

From the history of the epidemic of malaria, from the figures for kala-azar from the two hospitals and from the Graph, it will be seen that though 1939 was the year when malaria was most prevalent, it was not till September 1940 that a sharp rise in the number of kala-azar cases occurred, from this time when malaria was dying down till March when investigations ceased 170 of the 234 cases were found. It would thus appear that there was a local 'flare up' of kala-azar two years after malaria in epidemic form first appeared. With a disease like kala-azar such an interval would not be incompatible with a connection between the two events, and direct evidence of malaria may have been demonstrated in a larger proportion of the patients had investigations been undertaken earlier. A closer examination of the results will, however, show that there are certain factors not quite in keeping with the theory presented by Napier and Krishnan, particularly we think regarding the mechanism of dissemination of the infection from a sub-clinical focus, if by the term malaria is meant acute malaria.

From the table showing the degree of splenic enlargement it will be noted that in 158 cases the spleen was 3 inches or less below the costal margin, and in only 41 was the enlargement greater than 4 inches. If the size of the spleen is accepted as a satisfactory indication of the approximate duration of the disease, more than two-thirds of the cases were early ones of three to four months' duration, and it should be stated, their general appearance was not suggestive of a more prolonged illness.

Also if the period of investigation is divided into two parts from March to August, before the 'flare up', and from September onward, the 'flare up' period, it will be seen that up to September there were 164 cases of malaria and 64 of kala-azar. Among the 64 kala-azar cases there was an association with malaria in 10, in five before and five at the same time, but if the two cases Nos 6 and 7, which were diagnosed malaria in March and May 1940 and kala-azar in March and February 1941 respectively, be excluded on the score that malaria preceded kala-azar by too long an interval (the splenic enlargement in these cases being $2\frac{1}{2}$ inches and 1 inch respectively) and case No 9 is included among those which showed both diseases at the same time, the figures would read 3 before and 6 at the same time, a ratio of about 1 in 11 kala-azar cases with malaria parasites in their peripheral circulations. During the 'flare up' there were 93 cases of malaria and 170 of kala-azar, with an association of both diseases in five, two before and three at the same time, these three cases were all found in September when there was also a slight increase in malaria. For the 'flare up' period therefore the proportion of cases with malaria parasites in their blood smears would be about 1 in 56, or if September's figures be excluded, zero out of 139. The detection of malaria parasites in 1 of the 11 kala-azar patients is a higher rate than usual in hospital practice, which we believe to be more like 1 in 56. The difference in the ratios is significant and

would seem to vary directly with the amount of active malaria among the population, a condition not quite in keeping with the contention that malaria is suppressed by the histiocytosis accompanying kala-azar

The increase in the number of kala-azar cases in 1939 and early 1940 may have been due to the generalization of the disease by the action of malaria, in persons harbouring sub-clinical foci of infections as visualized by Napier and Krishnan, but the same cannot be said of these from September onward, if it is agreed that they were mostly early ones, their occurrence about the middle of the estimated period of the sandfly transmission of *L. donovani*, when relatively little active malaria was present, would indicate that they developed from infections contracted that season and without the doubtful aid of active malaria. The absence of malaria parasites in the blood smears of well over a hundred kala-azar cases examined after September, compared with results before that month, would also support this contention

The collection of sandflies from many of the dwelling houses in this area did not reveal the presence of abnormal numbers, and unless it be argued that there was an increase in the invasive powers of the strain of parasite present, the 'flare up' may be ascribed to an increased susceptibility of the population resulting from the epidemic of malaria. The administration of small doses of quinine just sufficient to control the severity of the symptoms, combined with the general attitude of the people who were reluctant to swallow a bitter mixture and could not understand why they were not all given injections for their febrile condition, tended to the production of a chronic infection rather than the eradication of the disease among a large proportion of them. The result of a house-to-house survey of this area by a malaria survey party working under the Bihar Public Health Department during the winter months revealed that there was enlargement of the spleen in about 41 per cent and parasites in nearly 19 per cent of 456 children of 10 years of age and less. Very few, if any, of these children were brought to the centre for treatment though they almost certainly suffered from occasional bouts of fever. It is very probable therefore that the 'flare up' was the result of chronic malaria among the people which lowered their natural resistance to general visceral leishmaniasis. In this connection it may be of interest to note that benign tertian malaria, which gives rise to a chronic form more often than malignant tertian infections, was present in 10 of the 15 cases in which both diseases were associated

The absence of malaria parasites from the peripheral circulations of most kala-azar patients from rural areas therefore may be ascribed not so much to the suppression of the plasmodial infection by the histiocytosis of kala-azar as to the paucity of parasites in the blood of chronic cases. It is also possible that the generalization of an infection with *L. donovani* results not only by the carriage of leishmania-laden cells from a sub-clinical focus in the skin to the visceral endothelial tissues, but also to the cells being more receptive to the development of the parasites owing to a certain degree of overloading with hæmoglobin

Although the circumstantial evidence in favour of malaria predisposing to kala-azar is so strong, direct evidence obtained so far is comparatively scanty

Both diseases are transmitted by insects which require very similar conditions of temperature and humidity for the development and propagation of their respective parasites. The fact also that practically every kala-azar area is one of malaria may account for examples of both diseases in the same patient either at the same time before or after each other. The detection of an association of both diseases in the same patient in only 15 of 234 cases, together with the occurrence of the 'flare up' when malaria was dying down, and the difference in the number of kala-azar cases showing malaria parasites in their blood at different periods relative to the epidemic of malaria, might mean that the association between the two diseases was merely regional, and whatever stimulus was responsible for the epidemic of malaria also influenced the 'flare up' of kala-azar.

Whether malaria predisposes to kala-azar or both diseases be activated by the same epidemic stimuli, from a practical point of view there is little doubt that in rural areas where both diseases are prevalent, measures of control directed against the adult forms of the vectors which shelter together in dwelling houses would be more economical and probably produce better results than measures to deal with their larval stages which are found in totally different environments and spread over wide areas.

ACKNOWLEDGMENT

In conclusion we would take this opportunity to express our thanks to Dr G Prasad, the District Health Officer, for his help and co-operation.

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FURTHER INVESTIGATIONS ON THE TRANSMISSION OF KALA-AZAR

Part VI

A SECOND SERIES OF TRANSMISSIONS OF *L DONOVANI* BY *P ARGENTIPES*

BY

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[Received for publication, June 20, 1941]

THE transmission of *L donovani* to hamsters by bites of infected sandflies was repeated in 1940 to determine whether the successful results obtained the previous year and reported in Part III of this series (Smith, Halder and Ahmed, 1940) were dependent on the new method of feeding and keeping the flies or on the virulence of the local strain of parasite with which the flies were infected. Parallel feeding experiments were therefore undertaken with two sets of flies maintained according to the old and new methods respectively. The flies were all bred under identical laboratory conditions and given preliminary infecting blood meals on the same patients in the same cages and not separated into different batches till three or four days later, such flies as oviposited and took a second blood meal on the same or another kala-azar patient were then placed in one category and given the third and subsequent feeds on experimental animals, as was the practice in former years, while the remainder were fed on raisins till the tenth day before being utilized to deliver infective bites. The object of the experiment was to prepare

the way for the transmission of *L donovani* by *P argentipes* to a human being and determine whether flies had to be infected from a particular type of case if a successful result was to be obtained

Transport difficulties due to war conditions prevented the importation of a fresh stock of hamsters from Palestine and only ten animals were available for the experiment. It was decided to give each hamster approximately ten known positive feeds, and exposure of the animals to infection was begun about the middle of June

Details of the experiment and the results of examination of these animals are given in Table I. Hamsters HB 1 to HB 5 were bitten by flies maintained according to the old method and RH 6 to RH 10 by raisin-fed flies

TABLE I

Serial number	Placed under experiment on	Total flies fed	Positive	Negative	Unknown	Date of examination	Result
HB 1	17-6-40	59	10	16	33	Lost	Negative
HB 2	3-7-40	63	10	12	41	18-2-41 Sacrificed	
HB 3	17-7-40	51	10	14	27	18-2-41 Sacrificed	
HB 4	29-7-40	119	15	45	59	18-2-41 Sacrificed	
HB 5	7-8-40	84	12	28	44	18-2-41 Sacrificed	Positive
RH 6	21-6-40	64	12	24	28	13-2-41 Liver puncture	
RH 7	12-7-40	20	9	4	7	16-2-41 Sacrificed	
RH 8	16-7-40	20	14	5	1	14-2-41 Liver puncture	
RH 9	19-7-40	26	11	3	12	18-2-41 Sacrificed	.
RH 10	25-7-40	85	13	34	38	14-2-41 Liver puncture	

All five hamsters bitten by flies maintained according to the new method were proved infected by the presence of parasites in fairly large numbers in the liver-puncture material or smears of liver and spleen tissue. Four animals exposed to bites from flies which were given repeated blood meals were all negative, it is unfortunate that one of these hamsters escaped after gnawing a hole in its cage and was not seen again, but the fact that the other four were negative even by cultural examination, combined with the numerous unsuccessful results in former years when this method of feeding was regularly practised, is sufficient evidence that the nature of the case from which the flies derive an infection does not make an appreciable difference in the transmission results.

Sixteen mice were also given varying numbers of bites from infected flies, excluding those which died before a sufficient incubation period had elapsed and which could not be tested by cultural methods, or examined satisfactorily under a microscope owing to decomposition changes, the results of twelve which were sacrificed are given in Table II —

TABLE II

Serial number	Placed under experiment on	Total flies fed	Positive	Negative	Un known	Date of sacrifice	Result	REMARKS
M 2	16-5-40	106	15	49	42	13-2-41	Negative	
M 3	9-5-40	63	11	32	20	18-2-41	"	
M 4	23-5-40	49	8	26	15	13-2-41	Positive	By culture
M 5	23-5-40	44	11	10	23	18-2-41	"	"
M 6	18-6-40	91	12	41	38	21-2-41	Negative	
M 8	27-5-40	51	10	18	23	20-2-41	"	
M 10	27-5-40	89	18	38	33	2-2-41	Positive	By culture
M 11	11-6-40	21	14	3	4	20-2-41	Negative	
M 13	16-6-40	46	11	15	20	20-2-41	"	
M 14	18-6-40	30	2	11	17	18-2-41	"	Feeding stopped as mouse pregnant.
MF 2	19-8-40	90	19	25	46	22-2-41	Positive	By culture
MF 3	31-8-40	78	9	33	36	21-2-41	Negative	

MF 2 and MF 3 were female mice which were caged with males after they were given a number of infective bites to determine whether pregnancy and lactation would influence the course of an infection with *L. donovani*, as neither mouse

produced a litter, they are classed among the others. Pregnancy however did not effect the result in the case of M 14 which had two positive feeds and later produced a litter. Four of the 12 mice which were sacrificed and examined were proved infected by cultural methods, this result is not as good as that obtained last year when the only two mice which survived the period of incubation were both found infected and one showed parasites in the smears of spleen tissue, the fact however that on this occasion as in the previous one it was not the mice which had the largest number of positive bites that were found infected would suggest that there possibly is a certain degree of individual variation in the susceptibility of mice to leishmaniasis.

To get an indication of the number of bites necessary to produce an infection, three hamsters were given one, two and three bites respectively from 'blocked' flies. The animal that had two bites died three months later and no parasites were found in smears of spleen or liver. The other two were sacrificed six and seven months after exposure to infection, cultures of the hamster bitten by one infected fly were negative, and those of the other were unfortunately contaminated. It is hoped to repeat this experiment when time and opportunity permit.

REFERENCE

SMITH, HALDER and AHMED (1940) *Ind Jour Med Res*, **28**, 2, p 585

THE TRANSMISSION OF *LEISHMANIA TROPICA*
BY THE BITE OF *PHLEBOTOMUS*
PAPATASII

BY

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AND

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[Received for publication, May 3, 1941]

THE following experiments were carried out —

1st January, 1941 Ten laboratory-bred sandflies *Phlebotomus papatasi* ♀ Nos 7 to 16 fed through a membrane on a suspension of flagellates *Leishmania tropica*. The flagellates were washed by centrifugation in 2.7 per cent saline, and the final suspension consisted of flagellates in 3 parts 2.7 per cent saline and one part inactivated de-fibrinated rabbit blood.

After feeding through the membrane the sandflies were placed in an incubator at 30°C and kept in test-tubes plugged with slightly moistened cotton-wool.

The relatively large concentration of saline in the initial feed, and in some cases also in the first re-feed, had no adverse effect either on the egg development or longevity of the sandflies.

The strain of *Leishmania tropica* used for these experiments was maintained on Locke blood-agar. Cultures were used by Dr L Katzenellenbogen for inoculation experiments on human beings and found infective.

The sandflies were taken out at various intervals and re-fed on a laboratory worker who had been infected with *Leishmania tropica* on 2nd November, 1925, by inoculation of flagellates from a wild sandfly caught in Jericho on 26th October, 1925. He was cured spontaneously and during the last 16 years had repeatedly fed laboratory-bred sandflies on himself for breeding purposes. During these years thousands of sandflies had fed on him and he had become completely immune to their bites which eventually produced only a transient local reaction on him.

On 9th January, 1941, all the ten infected sandflies re-fed on him (for the fourth time) and at least six of the bites were followed by a very distinct reaction indistinguishable from a Leishmanin reaction. It was therefore suspected that flagellates had been inoculated into his skin by sandflies which re-fed on him on 9th January, 1941.

By 12th January, 1941, all the sandflies except one (No 16) were dead. The remaining sandfly was then placed in an incubator at 23°C and re-fed on various days on a number of volunteers. It died on 23rd January, 1941.

In all other experiments the sandflies were kept at 30°C except for the short periods in which they were taken out to re-feed.

13th January, 1941. Five laboratory-bred sandflies *Phlebotomus papatasi* ♀ Nos 17 to 21 were fed on a culture of *Leishmania tropica* as above.

15th January, 1941. Sandfly No 17 re-fed on a culture as above.

27th January, 1941. Five laboratory-bred sandflies *Phlebotomus papatasi* ♀ Nos 22 to 26 fed on culture of *Leishmania tropica* as above.

29th January, 1941. Four sandflies Nos 23 to 26 re-fed on culture as above.

16th February, 1941. Twenty laboratory-bred sandflies *Phlebotomus papatasi* ♀ Nos 27 to 46 fed on a culture as above.

18th February, 1941. Two sandflies died, the remainder (Nos 29 to 46) all re-fed on culture as above.

27th February, 1941. Eleven laboratory-bred sandflies *Phlebotomus papatasi* ♀ Nos 47 to 57 fed on culture as above. (The re-feeds with the latter 11 sandflies are not included in this paper.)

All the sandflies used in these experiments were laboratory-bred ones raised from females caught in Jerusalem. They included individuals of the first and second generation bred in the laboratory.

Of the 31 sandflies Nos 16 to 46 two died 2 days, one 3 days, one 4 days, and one escaped 2 days after the infecting feed, the transmission experiments in this paper refer only to work with the remaining 26 sandflies. (The few sandflies dissected after 2 days were already found to have a heavy infection extending to the most anterior part of the cardia.)

Up to the time of writing—20th April, 1941—these 26 sandflies had produced 28 individual lesions on four volunteers on whom they re-fed repeatedly at various intervals. Twenty of these lesions were produced by no more (and possibly less) than nine sandflies and 27 of the lesions by no more than 11 sandflies.

The details are as follows —

Volunteer No 1 —

21st January, 1941. Sandflies Nos 20 and 21 fed on volunteer No 1 (i.e. 8 days after the infecting feed, during this period they re-fed on other volunteers).

Immediately after the feed a little fluid was seen emerging from one of the puncture wounds. This was smeared on a slide, stained and found to contain flagellates. Most of the flagellates removed with the fluid were obviously dead.

PLATE XIV



FIG 1 Lesion on volunteer No 1



FIG 2.



FIG 3

FIGS 2 and 3 Some of the lesions on volunteer No 2

On 17th March, 1941, the lesions extended and two ulcerated

Volunteer No 4 --

20th February, 1941 Sandflies Nos 32 to 46 fed on volunteer No 4

25th February, 1941 Sandflies Nos 45 and 46 fed on volunteer No 4

17th March, 1941 One papule was noted on a site on which sandflies fed and on examination numerous L-D bodies were found

Volunteer No 5 --

5th February, 1941 Sandflies Nos 24 to 26 fed on volunteer No 5

24th March, 1941 Two papules appeared on sites of bites of two sandflies Both contained L-D bodies

Volunteer No 6 --

31st January, 1941 Sandflies Nos 22 to 26 fed on volunteer No 6
No lesions have yet appeared

Volunteer No 7 --

25th February, 1941 Sandflies Nos 45 and 46 fed on volunteer No 7
No lesions have yet appeared

Volunteer No 8 --

21st February, 1941 Sandflies Nos 32 to 46 fed on volunteer No 8

23rd February, 1941 Sandflies Nos 39 to 46 fed on volunteer No 8

27th February, 1941 Sandfly No 46 fed on volunteer No 8

2nd March, 1941 Sandfly No 46 fed on volunteer No 8 Up to the present no lesions have appeared

Volunteer No 9 --

17th January, 1941 Sandfly No 16 fed on volunteer No 9 Up to the present no lesions have appeared

Other experiments involving re-feeds of another 11 sandflies have not yet been read off and we must wait some months before the final results of the experiments are known

Analysing these experiments we find --

Twenty-six sandflies produced 28 lesions after 45 individual feeds (of which at least four were not concerned with transmission) and of these 27 were produced by no more and possibly by less than 11 of the sandflies and 20 by no more and possibly by less than nine sandflies. The 11 sandflies produced 27 lesions after a total of 30 individual feeds (of which at least four were not concerned with transmission) but these 30 individual feeds represented much more than the same number of bites, for, as already pointed out, sandflies Nos 20 and 21 bit at least ten times before obtaining blood on 20th January, 1941. The two latter sandflies were probably concerned in the infection of more than one volunteer

(Nos 1 and 2) Two of the sandflies Nos 24 and 26 probably infected volunteers
 Nos 2 and 5

The information is summarized in the Table —

TABLE

Showing the results of feeding on five volunteers

Volunteer number	Number of infected sandflies fed.	Total number of individual feeds	Total number of lesions produced
1	2	2	1
2	9	11	18
3	5	14	6
4	15	17	1
5	3	3	2

N B—The number of individual bites is considerably greater than the number of individual feeds

REMARKS

(1) The experiments were carried out with laboratory-bred sandflies which hatched out at a time when there were no adult sandflies in nature. The sandflies were raised from eggs laid in the laboratory by wild sandflies and descendants of wild sandflies caught in Jerusalem.

(2) The experiments were carried out on human beings not exposed to natural infection during the whole course of the experiment.

(3) The lesions occurred on the exact site of bites of the sandflies used in the experiment.

The above are therefore reasonably controlled experiments in which *Leishmania tropica* was transmitted by the bites of sandflies.

It is also obvious that in the above experiments transmission of *Leishmania tropica* by the bite of laboratory-bred sandflies was accomplished with almost ridiculous ease as compared to previous attempts.

In previous experiments (Adler and Theodor, 1929) 253 sandflies *Phlebotomus papatasi* infected with *Leishmania tropica* re-fed repeatedly on 12 human beings and a puppy. Only one human being became infected on a site on which sandflies had fed but it was thought advisable not to consider this experiment conclusive because the volunteer in this experiment had lived for some time in a quarter where a few cases of oriental sore had occurred and natural infection was

not absolutely excluded. The numerous definitely negative experiments were impressive and required elucidation.

In numerous other unpublished experiments carried out in 1929, laboratory-bred sandflies were infected either by feeding on the tails of mice on lesions swarming with L-D bodies or on cultures, and they were re-fed repeatedly on volunteers with a negative result.

Later (1931 and 1935), after studying infections of *L. infantum* in *P. perniciosus* the conclusion was reached that sandflies deposit flagellates into any medium on which they feed if and only if the distal part of the epipharynx is infected. We have seen no evidence of ejection of flagellates from the pharynx or buccal cavity. The motility of the flagellates is not essential for their deposition from the distal end of the epipharynx because we have noted the deposition of both active and inactive flagellates by *P. perniciosus*.

The negative result previously reported may be due either to loss of infectivity of *Leishmania tropica* in the sandfly, or to the fact that flagellates were not deposited into the skin during the act of piercing. Human beings have been repeatedly infected in the laboratory with *Leishmania tropica* by flagellates from dissected sandflies *Phlebotomus papatasi* both naturally and artificially infected (Adler and Theodor, 1925, 1926, 1927) and we therefore are of the opinion that the negative results of feeding experiments were in many cases due to non-entrance of parasites into the skin, i.e. flagellates did not invade the distal part of the epipharynx.

The factors which determine the invasion of the epipharynx by flagellates are not known, but it seems that intensity of infection in the sandfly though important is not the only one. The epipharynx is not invaded in many sandflies with infections so intense that numerous flagellates escape into the coelome. The chitinous inferior surface of the epipharynx is not indifferent and in many sandflies acts as an effective barrier to the forward passage of flagellates, although there is no anatomical barrier. In an unpredictable number of sandflies flagellates pass readily down to the distal end of the epipharynx.

It seemed probable that physiological conditions in the sandfly determine the properties of the inferior surface of the epipharynx and that in *Phlebotomus papatasi* these might in the laboratory be influenced among other things by temperature and salt content. (We were very much impressed by the fact that in the Jordan Valley where *Phlebotomus papatasi* is the only transmitter of *Leishmania tropica* the disease is very common near the Dead Sea where the soil has a higher salinity and presumably the food of the larval stages of the sandfly contains more salt than in the northern part of the valley where oriental sore is rare. In a recent settlement near the Dead Sea 90 out of 120 new settlers became infected within 9 months, while in the northern part of the Jordan Valley no cases have occurred among hundreds of settlers.)

The positive experiments recorded in this paper differed from the previous negative ones only in two points —

- (1) The sandflies were kept at a constant temperature of 30°C.

(2) The sandflies were infected by feeding on flagellates suspended in 3 parts 2 7 per cent saline and one part de-fibrinated blood instead of in normal saline and de-fibrinated blood

Whether these factors were responsible for the positive results or whether the strain used was readily transmissible remains to be determined in further experiments

It is of course obvious that the experiments were carried out under artificial conditions and it remains to be determined how far they reflect natural ones

SUMMARY

Sandflies *Phlebotomus papatasi* were infected with *Leishmania tropica* by feeding on flagellates suspended in 3 parts 2 7 per cent saline and one part de-fibrinated blood and after feeding were kept at a temperature of 30°C

The sandflies re-fed on nine human beings of which five have so far become infected

Twenty-six infected sandflies were involved in the production of 28 individual lesions among the five volunteers, 18 of the lesions occurred on one volunteer on whom nine sandflies had fed two lesions on a volunteer on whom three sandflies had fed, one lesion on a volunteer on whom two sandflies had fed, six lesions on a volunteer on whom five sandflies had fed, and one on a volunteer on whom 15 sandflies had fed. (As seen in the text, a number of individual sandflies fed on more than one volunteer)

Not more than nine sandflies were responsible for 20 and more than 11 sandflies for 27 of the lesions after 30 individual feeds distributed among four volunteers, but these 30 individual feeds involved considerably more than 30 bites

Leishmania tropica has been transmitted to man by the bite of *Phlebotomus papatasi*

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NOTE ON AN INTRACELLULAR STAGE OF *LEISHMANIA CHAMELEONIS*, WENYON 1921

BY

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[Received for publication, May 3, 1941]

WE examined the stools of ten specimens of *Chameleo vulgaris* and found flagellates, *Leishmania chameleons*, in four. Of the six negative animals, five became infected after a single rectal injection of faeces containing *Leishmania chameleons* and one after introducing a small amount of infected faeces into its mouth. The latter animal died six days after infection and numerous flagellates were found both in the small and large intestines.

The parasites we studied call for no special description since they did not differ in any respect from those observed by Wenyon (1921) except in one case described below —

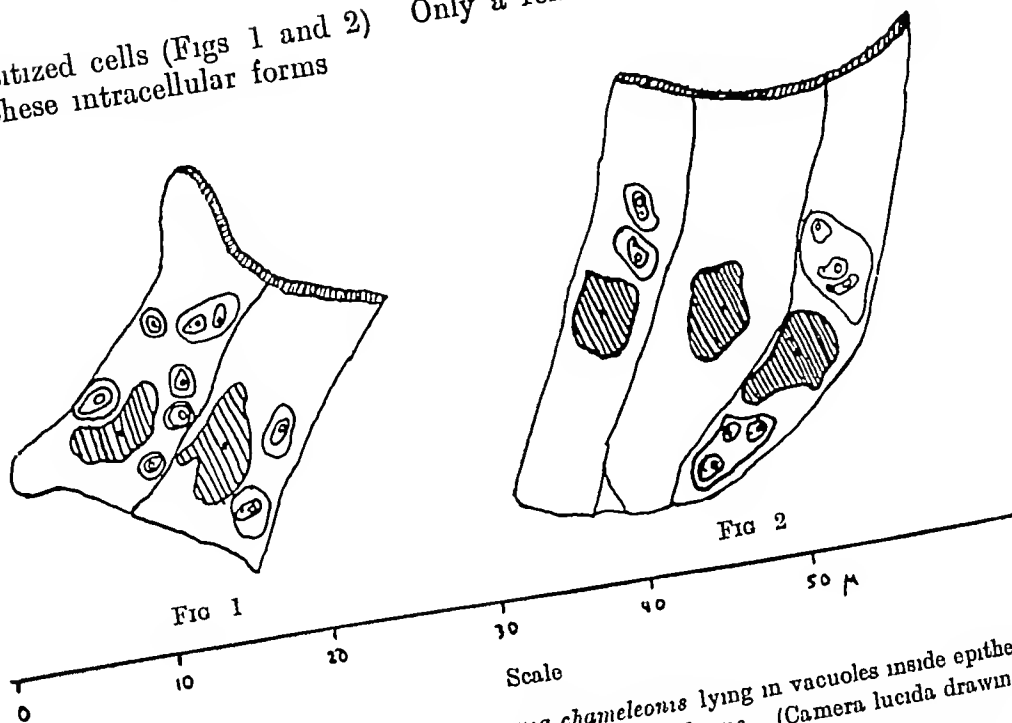
A chameleon which had been kept in the laboratory for six weeks was injected rectally with faeces (diluted with saline) containing *Leishmania chameleons*.

On the following day active flagellates of *Leishmania chameleons* were found in the faeces. Five days later heart's blood was sown on two tubes Locke serum agar and no flagellates were observed in the tubes during an observation period of two weeks.

Two weeks after the initial rectal injection the animal appeared moribund and was sacrificed. Sections of all parts of the large intestine showed flagellates in the lumen in close proximity to the epithelium. In some parts flagellates had invaded the mucosa where they did not appear to be ingested by any cells. A little above the cloaca *Leishmania chameleons* was found inside epithelial cells (Fig 1). The parasites had the form of L-D bodies and were enclosed in vacuoles in the cytoplasm of the epithelium, one or two parasites lying inside each vacuole. They were situated both distally and proximally to the nucleus of

Intracellular Stage of *Leishmania chameleonis*

the parasitized cells (Figs 1 and 2) Only a relatively small patch of intestine showed these intracellular forms



FIGS 1 and 2 —Flagellar forms of *Leishmania chameleonis* lying in vacuoles inside epithelial cells of the large intestine of *Chameleo vulgaris* (Camera lucida drawing)

This is so far the only record of a *Leishmania* invading epithelial cells of the large intestine

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INVESTIGATIONS INTO THE EPIDEMIOLOGY OF EPIDEMIC DROPSY.

Part XIII.

APPLICATION OF THE BIOLOGICAL TEST TO MODIFIED ARGEMONE OIL AND ITS DERIVATIVES

BY

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[Received for publication, May 22, 1941]

IN Part XI of this series (*vide Lal et al*, 1941) an account was given of a biological test for determining the toxicity of samples of oil. Although the results obtained justified the use of the method for this purpose, certain important information regarding the animals used was lacking which made it necessary to make some assumptions. Thus, in the absence of the knowledge of the age of the rat at the commencement of the experiment it was necessary to assume that the rodent had hitherto followed the growth curve according to Donaldson's experience and subsequent deviation, if any, could be ascribed to the experimental treatment

This assumption overlooked the possible influence of the different basic diets or of the different environmental conditions to which these animals had been subjected as compared with Donaldson's stock. Moreover, the duration of some of the experiments was not quite adequate. To remedy these deficiencies but primarily to determine the toxic properties of certain derivatives of toxic oils and of modified oil produced by the action of some physical agents or by extraction of certain fractions, a further series of experiments was carried out. For details of the experimental procedure and for the methods of appreciating the results reference should be made to the communication named above.

MATERIAL USED

Rats—The strain of rats used was the same as employed in the previous series. The age of the rodents which was definitely known, varied from one to two months at the commencement of the experiments. Before administering the experimental treatment, the animal was kept for sometime on basic diet. In a number of cases it was possible to use animals from the same litter for the experimental and control groups. The treatment was, as a rule, continued for two months or more.

GROUPS OF ANIMALS ACCORDING TO THE NATURE OF THE MUSTARD OIL USED

(1) *White crystalline substance (w c s)* added*

(a) GROUP I—Fed on solution of w c s in pure mustard oil equivalent to a 10 per cent concentration of argemone oil. Thus, 1 c c of the solution contained 0.20 mg of w c s. The group consisted of a pair of male and female rats.

(b) GROUP II—Given daily $\frac{1}{2}$ c c by intramuscular and subsequently by subcutaneous injections of w c s dissolved in olive oil to give a concentration of 0.40 mg per c c. The two rodents included in this group were both males.

(2) *Crystalline free base (c f b)**

GROUP III—Fed on solution of c f b in pure mustard oil, 1 c c contained 0.195 mg which is equivalent to about 10 per cent argemone oil. The group consisted of two male and two female rats.

(3) *Residue of argemone oil after abstraction of c f b dissolved in pure mustard oil*—Ether still remaining in the solution was driven off by gentle heating in a water-bath and by passing a current of air. The last traces of c f b were removed by repeated washings with water. To eliminate moisture fused calcium chloride was used. The extinction coefficient of the final product (after diluting

* For details see Part XII of this series (Mukherji *et al.*, 1941)

with pure mustard oil to obtain 10 per cent concentration) was reduced to 0.46 which was equivalent to 0.4 per cent of argemone oil

(a) GROUP IV —Fed daily with 0.015 c.c. to 0.15 c.c. of the residue. A pair of male and female rats was fed on this product. A third rat originally included in this group died in a fortnight.

(b) GROUP V —Fed on the same material to which w.c.s. had been added to obtain a concentration corresponding to 10 per cent argemone oil. It comprised of one male and two female rats. The object of this treatment was to test the hypothesis that the toxic agent in the argemone oil was a compound containing two radicals. One of these was abstracted in the form of c.f.b. (which is practically identical with w.c.s.) and the other remained behind and was liable to be converted into the original toxic compound on addition of w.c.s. to the residue.

(4) *Argemone oil modified by treatment with light*

Argemone oil exposed to direct sunlight for 50 hours was thus reduced to 9 per cent of the original.

GROUP VI —Fed on the modified oil diluted with pure mustard oil to the extent of 10 per cent. The group initially consisted of one male and one female rats but the former died in 18 days.

(5) *For purposes of control the following materials were used —*

Argemone oil control

GROUP VII —Put on diet containing 10 per cent argemone oil in pure mustard oil. This group comprised of one male and one female rats.

GROUP VIII —Fed on pure mustard oil. There were three male and two female rats in this group.

GROUP IX.—Consisting of one male and one female rats was maintained on the basic diet of the same composition as used in previous experiments.

The results obtained are set out in Table I, and in Charts 1-a to 9-b (*vide Appendix* for mathematical discussion of growth curves).

DISCUSSION

The histological findings would indicate that like the argemone oil, w.c.s. and to a certain extent c.f.b. exerts poisonous effect on rats when added to the food, but given subcutaneously w.c.s. does not manifest such a result, the swelling of the feet might have been due to local mechanical effect. The weight charts support the above evidence in regard to w.c.s., but not with regard to c.f.b. Argemone oil after removal of c.f.b. or modification through exposure

to light with loss of reactivity to chemical and physical tests, becomes biologically inactive in so far as may be inferred from the histological evidence. Unfortunately the experiment could not be continued for a sufficiently long period to follow changes in the weight curves with confidence though the available data show retardation in growth. As may be expected addition of w c s in the former case brings back the poisonous properties. Barring slight evidence of dilatation and congestion of capillaries in one or two observations mustard oil by itself does not cause any poisonous effects and the curves of growth though slightly lowered correspond to those of the rats put on the basic diet.

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APPENDIX

TABLE I

Details of experiment and histological findings

Nature of derivatives tested.	Rat number	Sex	Age (days)	Treatment (days)	Total consumption of oil (g) \equiv derivatives (mg)	HISTOLOGICAL CHANGES IN SKIN SECTIONS			REMARKS
						Number of days since commencement of feeding	Now capillaries	Dilated capillaries	
Group I White crystalline substance (w c s.) feed	150	♂	38	70	58.5 \equiv 11.7	42	+	+	Kept on basic diet for 28 days after the experiment
						57	+	+	
						75	+	+	
	151	♀	38	70	52.2 \equiv 10.4	15	+	+	Put on basic diet for 36 days after the experiment
						42	+	+	
						75	+	+	
Group II White crystalline substance (w c s.) injection	140	♂	60	50	23 \equiv 10.1	10	-	+	Dropsical oedema appeared in both hind feet
						24	-	+	
						32	-	-	
	141	♂	75	63	28.4 \equiv 12.7	15	-	-	
						32	-	-	
						72	-	-	
						93	-	-	

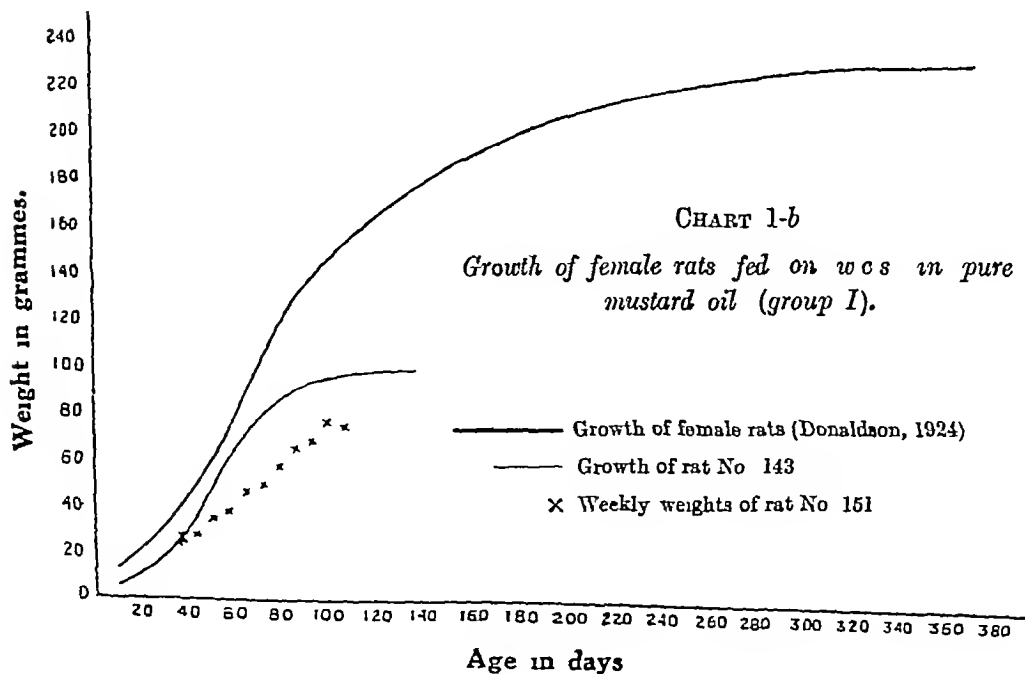
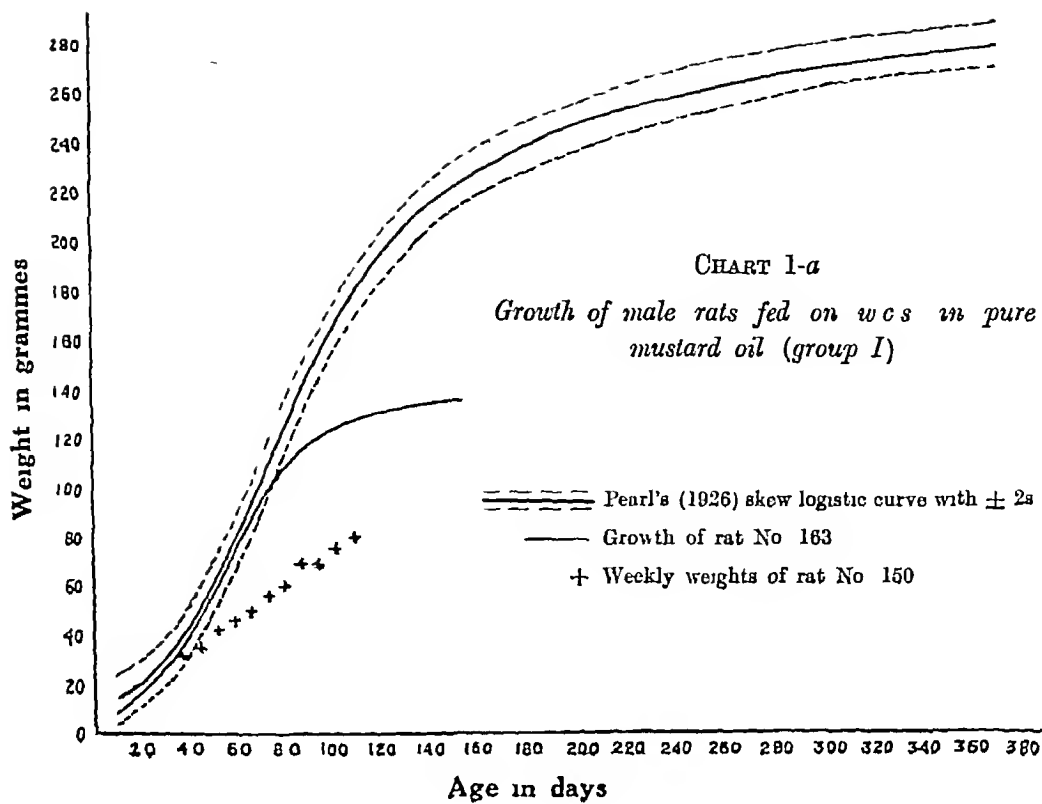
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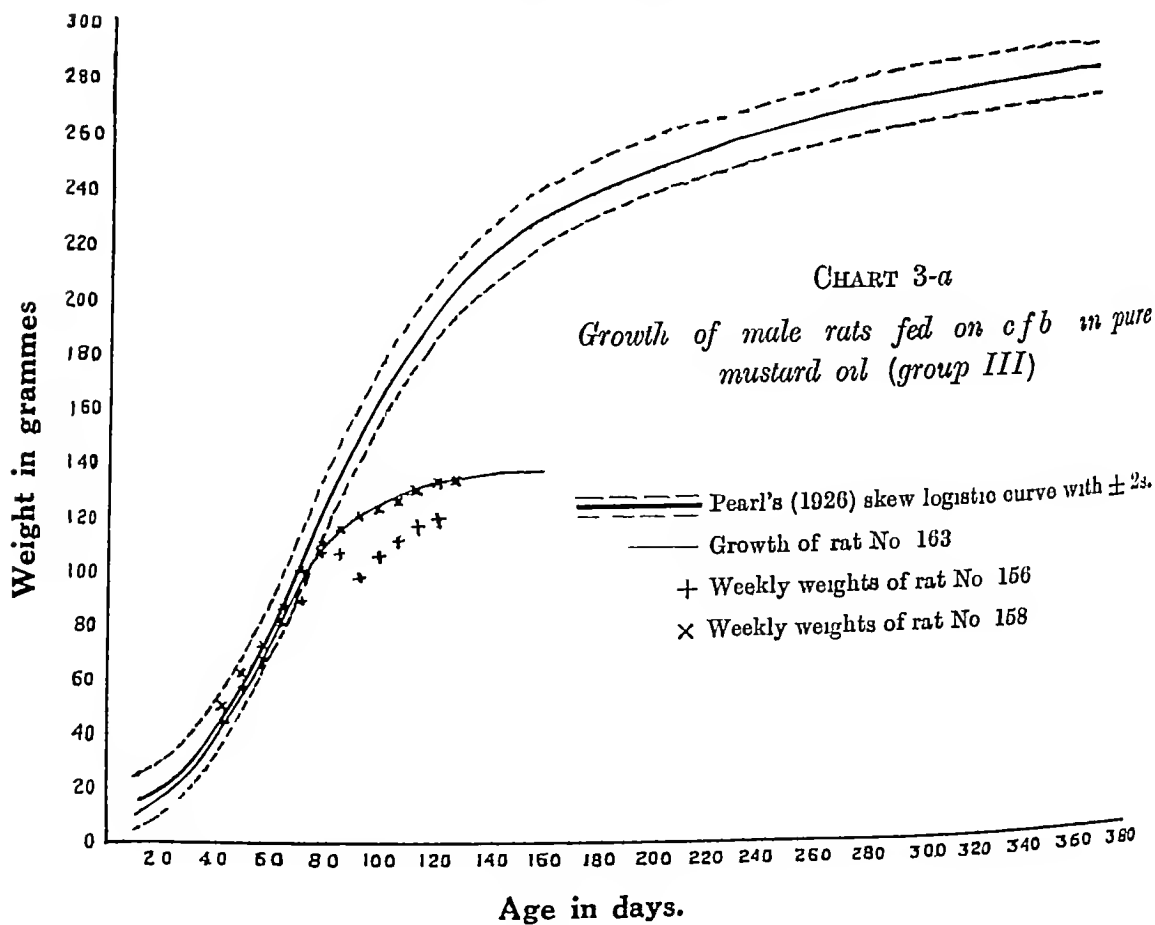
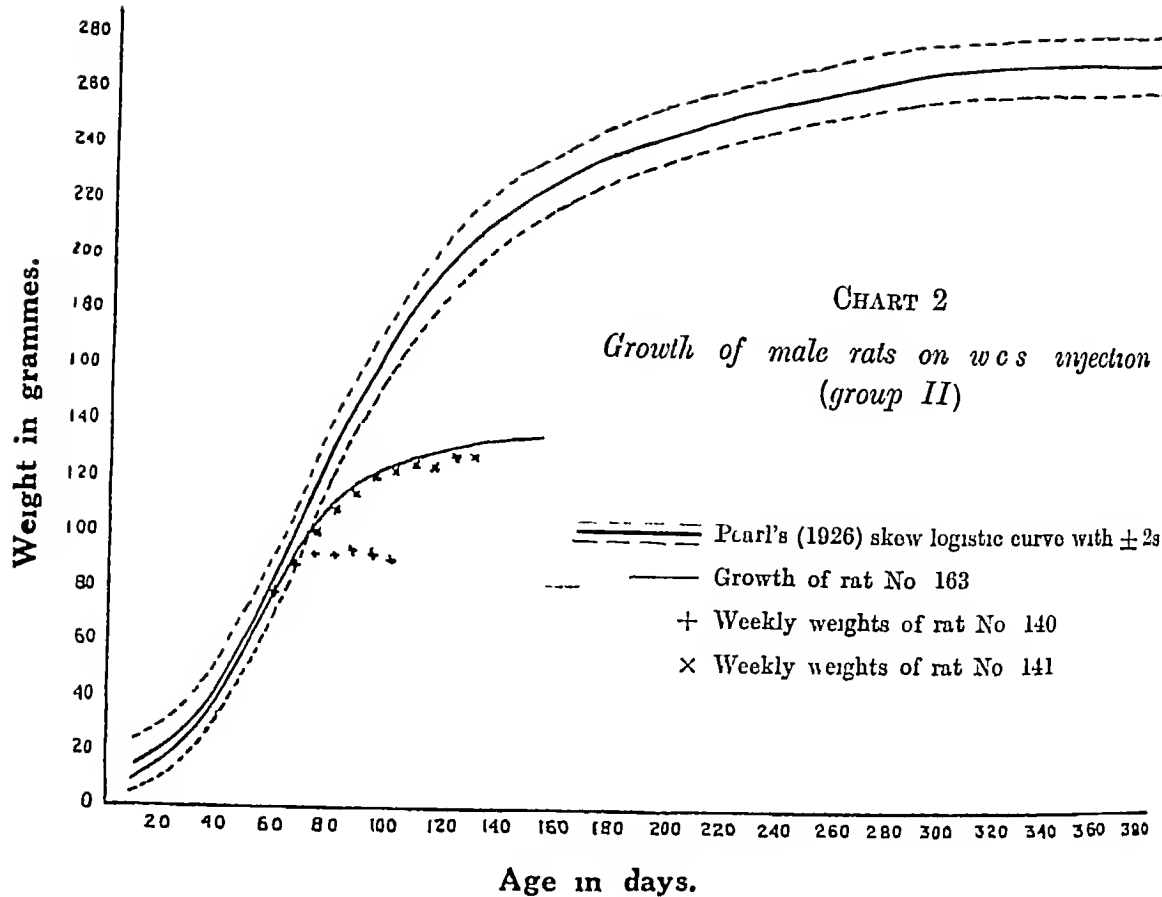
Nature of derivatives tested	Rat number	Sex	Age (days)	Treatment (days)	Total consumption of oil (g) ≡ derivatives (mg)	HISTOPATHOLOGICAL CHANGES IN SKIN SECTIONS			REMARKS
						Number of days since commence ment of feeding	New capilla-ries	Dilated capilla-ries	
GROUP III Crystalline free base (cfb) feed	156	♂	44	83	121.6 ± 23.7	15	+	+	Later sections were not satisfactory for examination
						31	+	+	
						49	+	+	
	157	♀	44	83	108.6 ± 21.2	15	-	-	
						31	-	+	
						49	+	+	
	158	♂	44	83	112.6 ± 22.0	15	+	+	
						31	±	±	
						49	+	+	
	159	♀	44	84	100.6 ± 19.6	15	+	+	
						31	+	+	
						49	+	+	

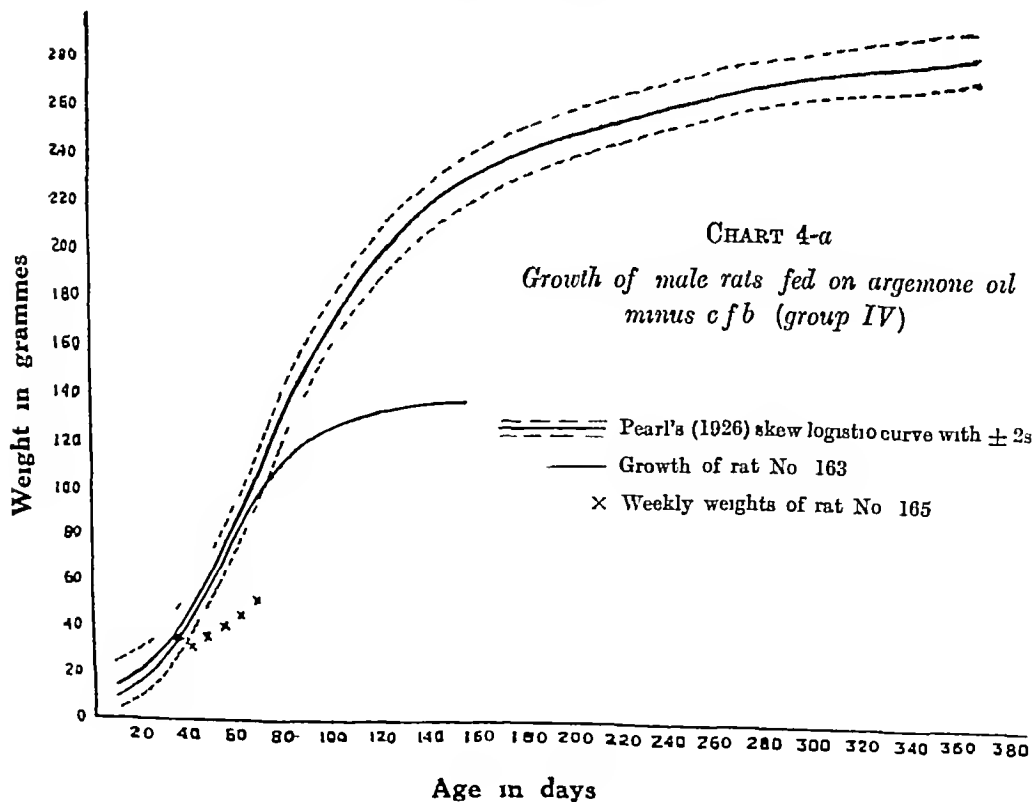
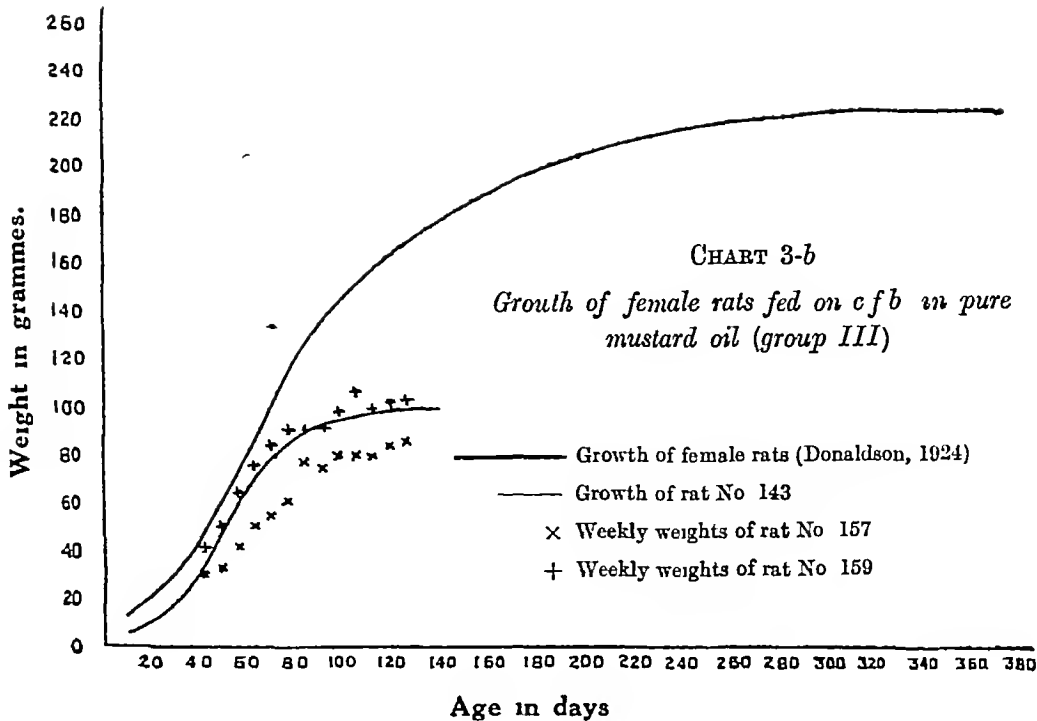
Group	Sex	Age	Weight (g)	Food (g)	Water (g)	Urine (g)	Feces (g)	Excreta (g)	Excreta (g)	Excreta (g)	Excreta (g)
Group IV Argemone oil minus cf b feed	♂	105	35	30	30.7	{	0	-	-	-	-
	♀	166	42	30	41.7	{	31	-	-	-	-
	♂	105	35	30	30.7	{	0	-	-	-	-
	♀	166	42	30	41.7	{	30	-	-	-	-
Group V Argemone oil minus cf b plus w c.s. feed	♀	166	20	73	82.2 \equiv 16.4 mg of w o s	{	28	+	+	+	+
	♂	170	20	73	70.0 \equiv 15.2 mg of w o s	{	42	+	+	+	+
	♀	171	20	73	67.5 \equiv 13.5 mg of w o s	{	50	+	+	+	+
	♂	171	20	73	67.5 \equiv 13.5 mg of w o s	{	73	+	+	+	+
Group VI Modified (by exposure to light) argemone oil feed	♀	168	42	40	40.3	{	0	-	-	-	-
	♂	168	42	40	40.3	{	17	-	-	-	-
	♀	168	42	40	40.3	{	40	-	-	-	-

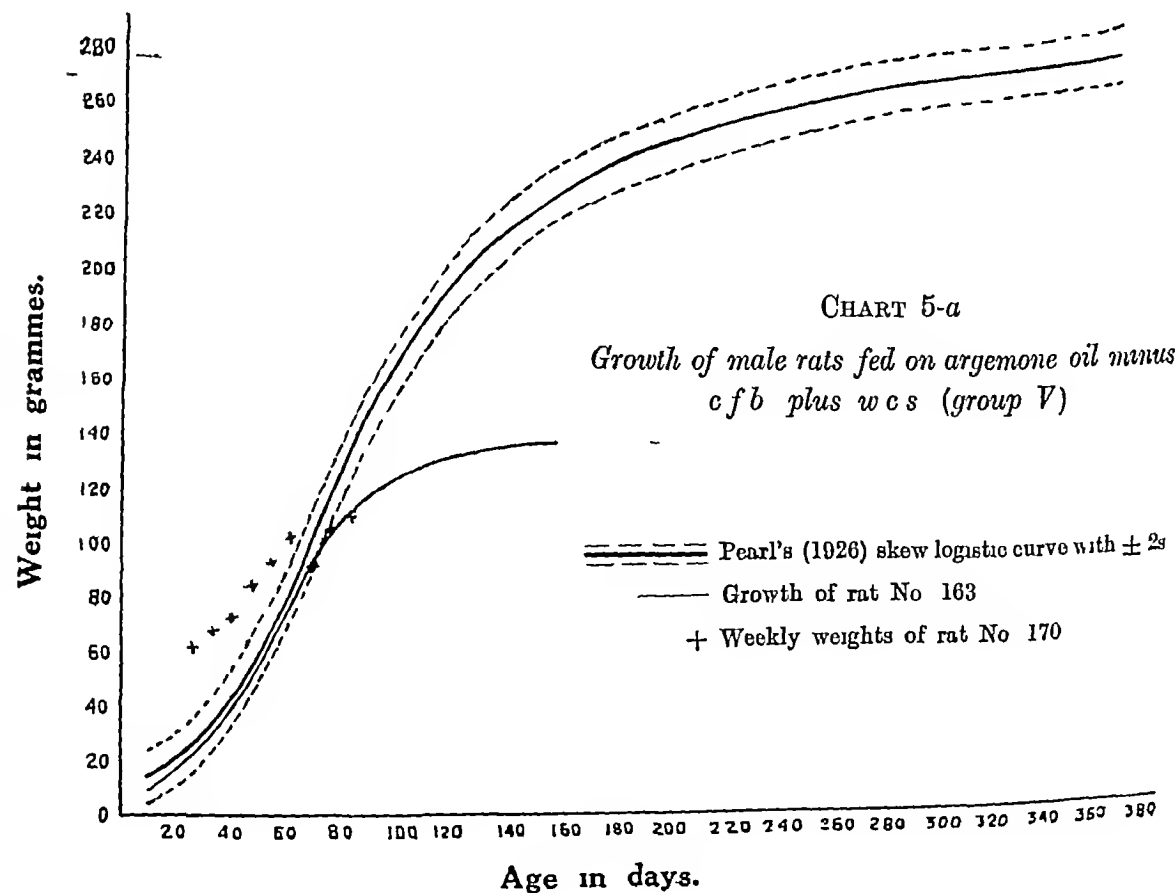
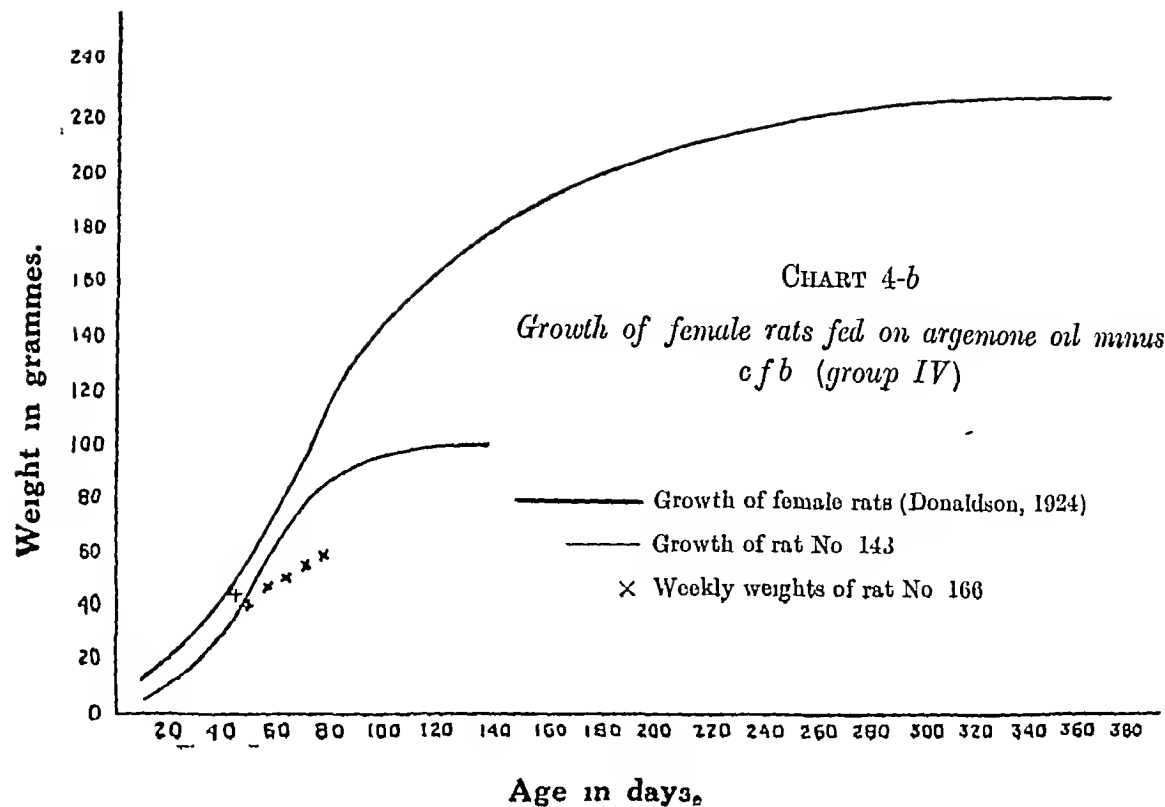
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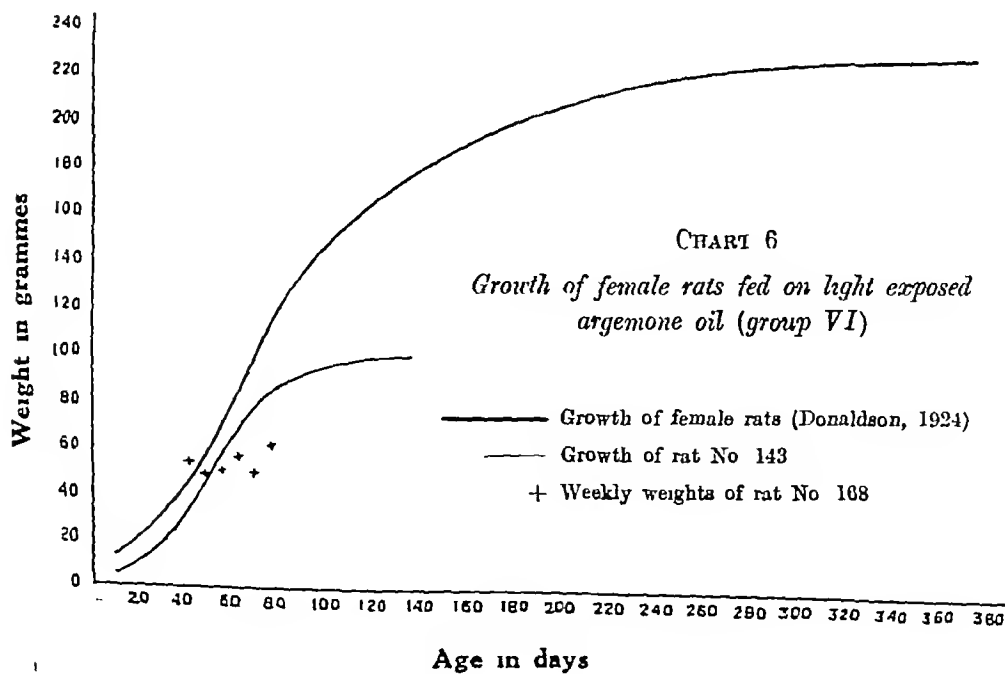
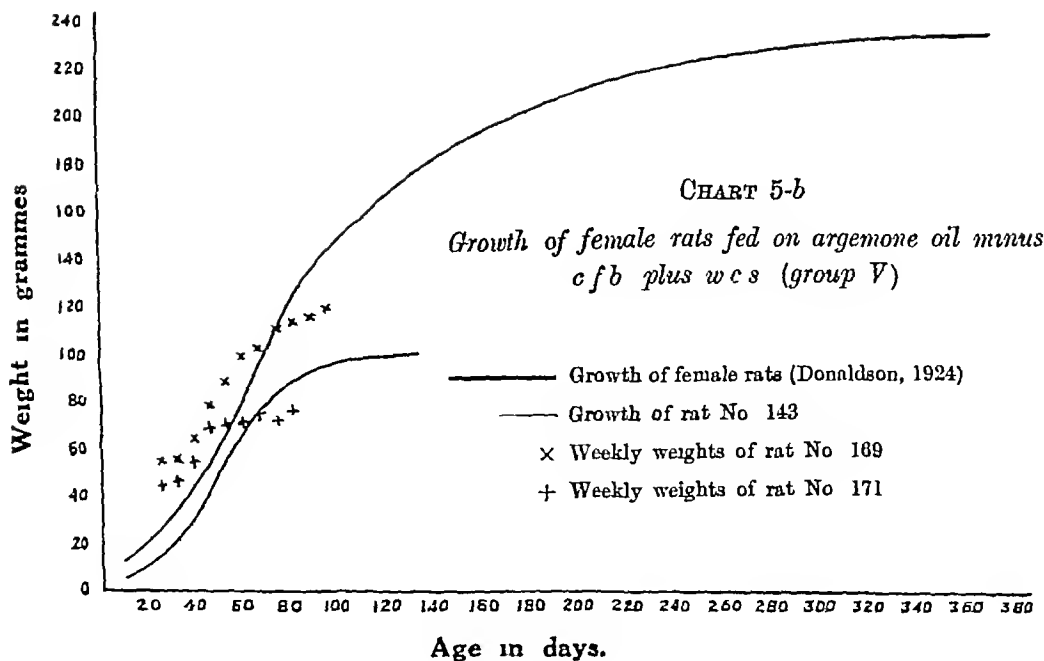
Nature of derivatives tested	Rat number	Sex	Age (days)	Treatment (days)	Total consumption of oil (g) \equiv derivatives (mg.)	HISTOPATHOLOGICAL CHANGES IN SKIN SECTIONS			REMARKS
						Number of days since commencement of feeding	New capillaries	Dilated capillaries	
GROUP VII Mixture of argemone oil feed	146	♂	38	70	40.2	58	+	+	Kept on basic diet for 30 days after the experiment
						102	+	+	
	147	♀	38	70	68.4	30	+	+	
						104	+	+	
GROUP VIII Pure mustard oil feed	144	♂	38	70	50.8	58	—	—	Kept on basic diet for 30 days after the experiment
						71	—	—	
						102	+	+	
	145	♀	38	70	58.2	0	—	—	Put on basic diet for 49 days after the experiment
						123	—	—	
	160	♂	44	85	103.8	85	—	—	
	161	♀	44	96	91.8	96	+	+	
	162	♂	44	96	106.5	96	—	—	
GROUP IX Basic diet feed	143	♀	38	98	Nil	43	—	—	
						78	—	—	
	163	♂	46	57	Nil	98	—	—	
						57	—	—	

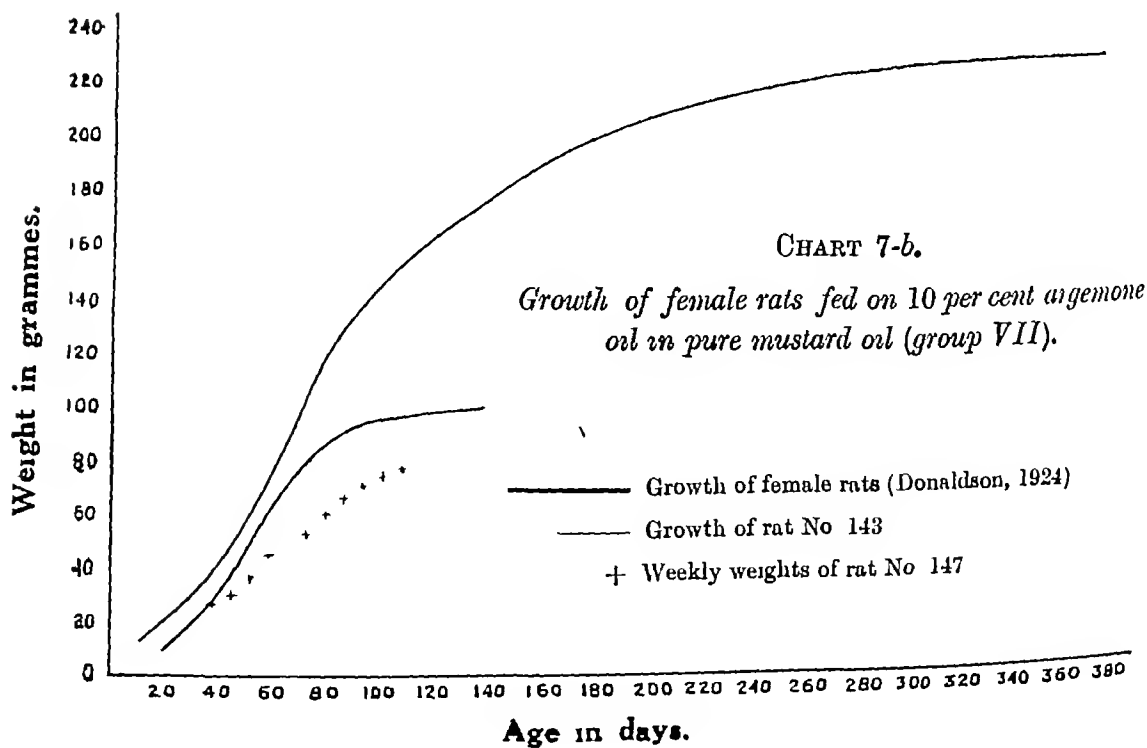
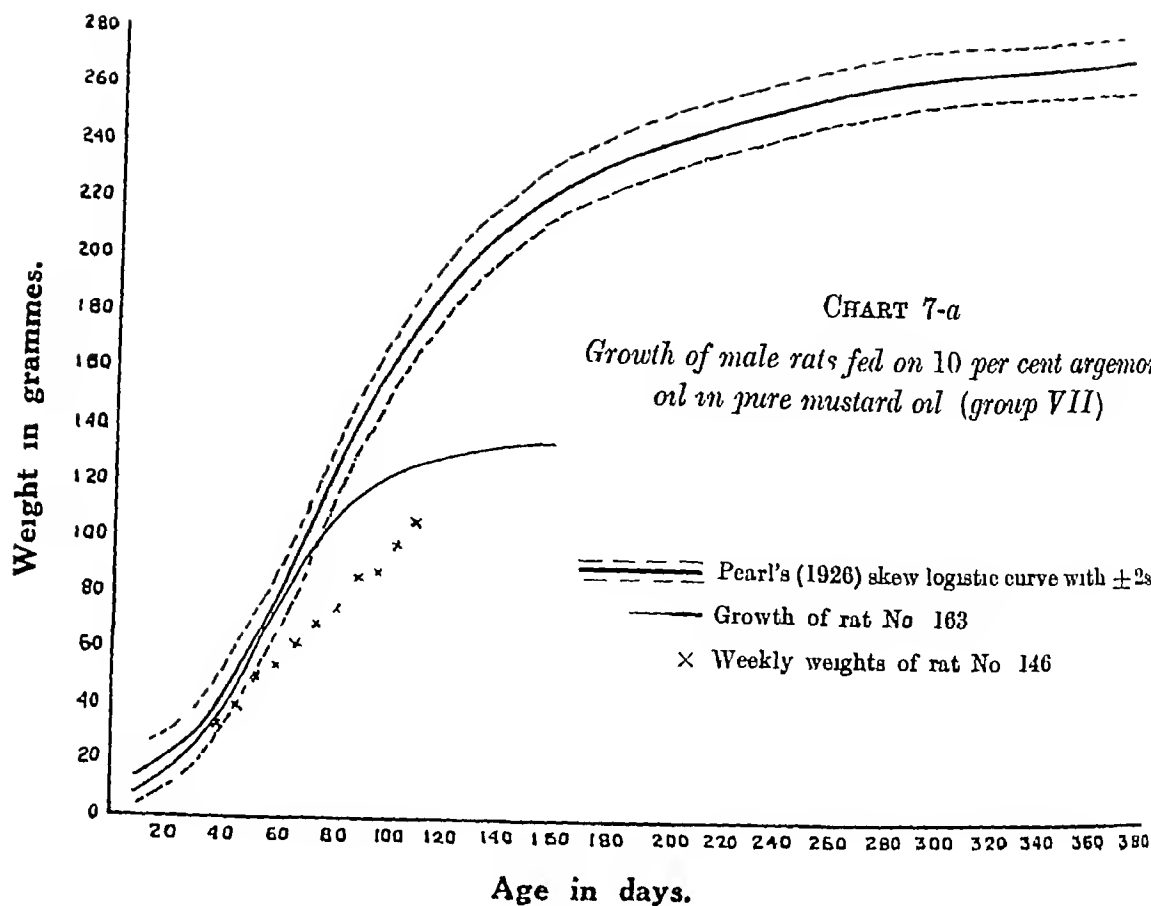


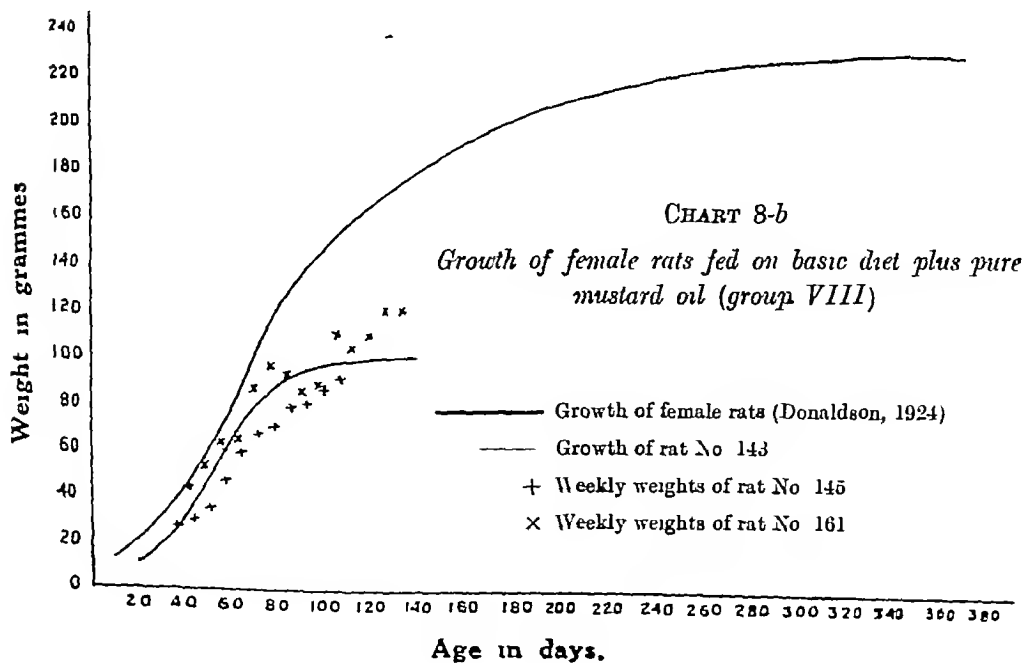
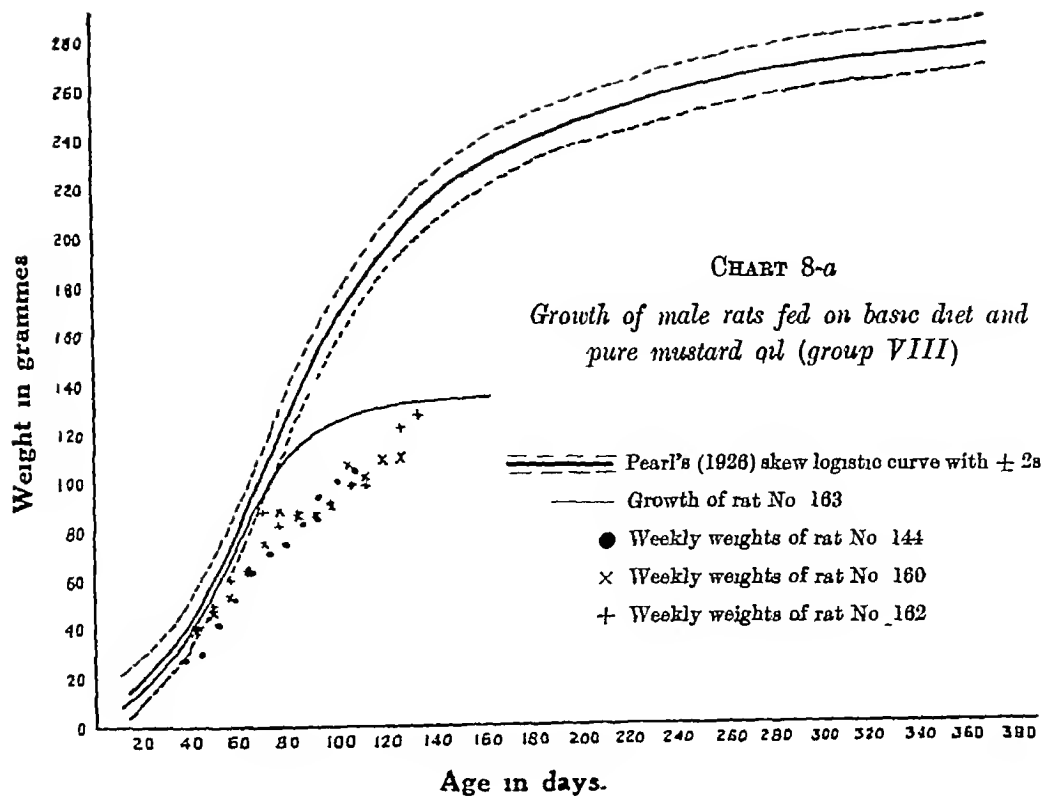


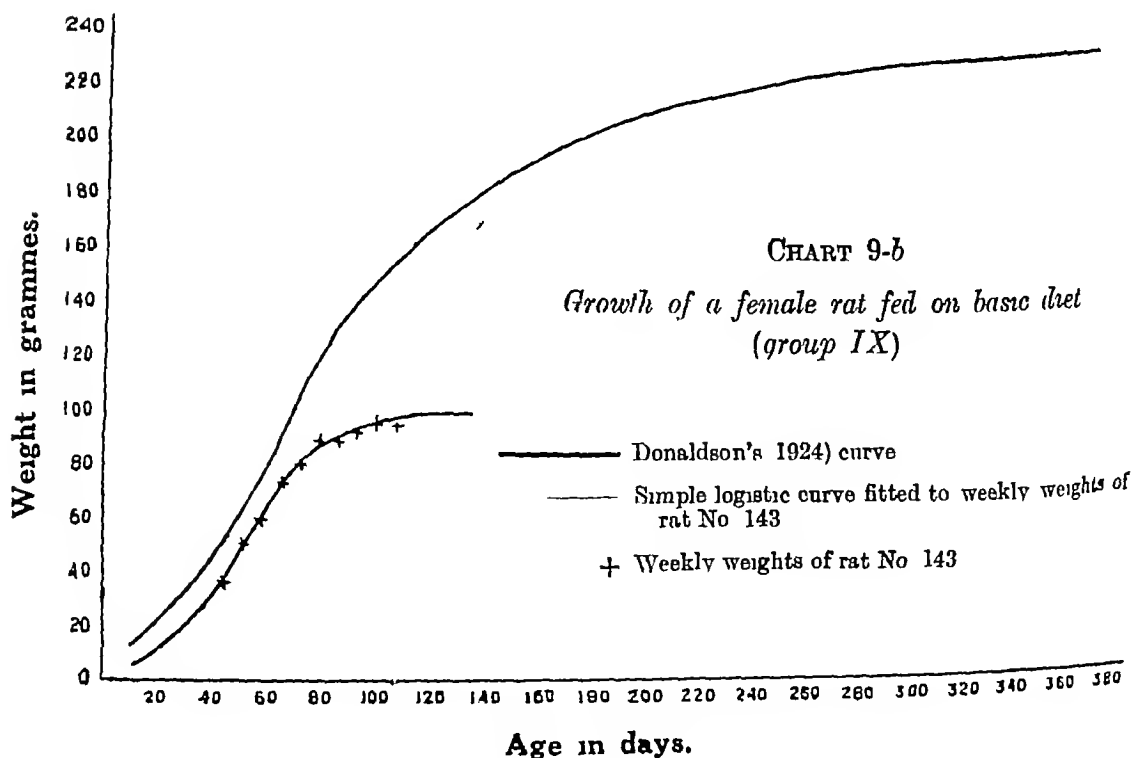
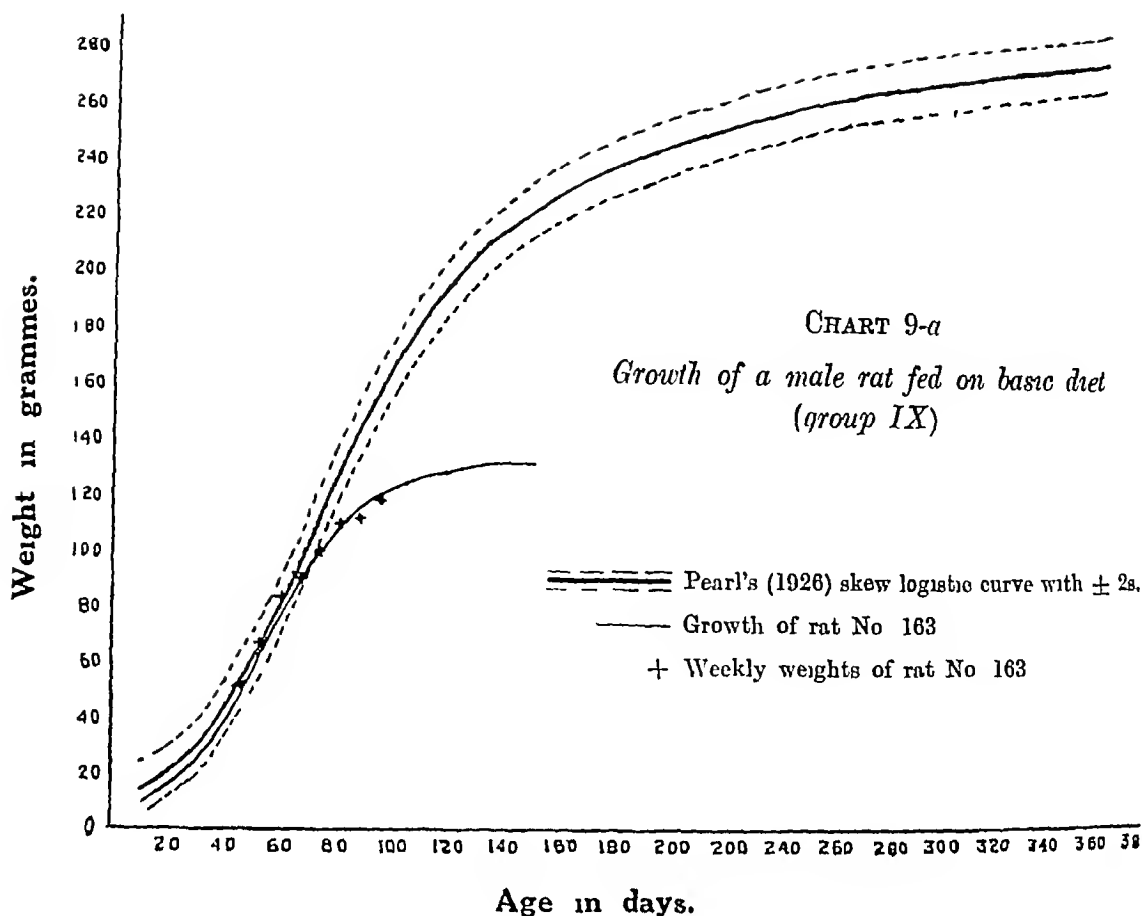












The statistical method of approach for testing whether the rats put on different treatments followed the logistic curve of growth was outlined in a previous communication. It was shown that the logistic curve gets transformed into a straight line if relative growth rate is plotted against weight.

Table II below gives the relative growth rates of individual rats against their weight.

TABLE II

The relative growth rates of rats against weekly weights

Group	Rat number	Sex.	Weekly weight (g)	Relative growth rate per cent per day	Group	Rat number	Sex.	Weekly weight (g)	Relative growth rate per cent per day
I	150	♂	40	2.96799	II	141	♂	108	0.93593
			50	2.14363				114	0.75257
			54	1.53653				120	0.48446
			62	1.75089				122	0.29160
			69	1.35967				125	0.11614
			75	1.73739				124	0.16941
			88	1.91059				128	0.22679
			98	1.32931					
I	151	♀	28	1.85366	III	156	♂	57	3.00361
			35	2.18133				67	2.51000
			38	2.10573				81	2.02819
			47	1.96027				89	1.92137
			50	1.50211				106	1.24861
			58	1.87404				106	-0.63379
			65	1.24047				97	-0.06771
			69	1.21016				105	0.96300
			77	0.52416				111	0.71166
II	140	♂	88	1.17914				116	0.43681
			92	0.31751					
			92	0.15349					
			94	0.00000					
			92	0.31061					

TABLE II—*contd*

Group	Rat number	Sex	Weekly weight (g)	Relative growth rate per cent per day	Group	Rat number	Sex	Weekly weight (g)	Relative growth rate per cent per day
III	157	♀	33	2 40339	IV	165 166	The period of observation was short		
			42	3 10944					
			51	1 92820	V	169	♀	56	1 08251
			55	1 27893					
			61	2 40339	V	169	♀	56	1 08251
			77	1 47581					
			75	2 73007					
			80	0 46100					
			80	0 00000					
			80	0 34850					
III	158	♂	62	2 70314				64	2 36686
			73	2 41981				78	2 27469
			87	2 24794				88	1 63043
			100	1 61031				98	1 04027
			109	0 99831				102	0 82510
			115	0 68674				110	0 73154
			120	0 48037				113	0 31751
			123	0 29160				115	0 36956
			125	0 34021	V	170	♂	62	1 79239
			129	0 38921				68	1 83251
			132	0 21811				73	1 86332
								85	1 92942
								93	1 96848
								102	2 00760
								91	1 95901
								105	2 02119
III	159	♀	51	3 11943				110	2 04139
			65	2 84937				113	2 05308
			76	1 91620				114	2 05690
			85	1 28661					
			91	0 48720					
			91	0 07807					
			92	0 60187					
			99	1 07887					
			107	1 03837					
			100	-0 27216					

TABLE II—*contd.*

Group	Rat number	Sex	Weekly weight (g)	Relative growth rate per cent per day	Group	Rat number	Sex.	Weekly weight (g)	Relative growth rate per cent per day
V	171	♀	47 55 69 70 71 75 72 76 79	1 43337 2 74266 1 72260 0 20410 0 49281 0 09091 0 09461 0 66273 0 54277	VIII	144	♂	30 42 52 63 71 75 83 94 101	2 89620 3 92893 2 89620 2 22456 1 24537 1 11544 1 61293 1 40201 0 79047
VI	168	♀	Period of observation was short.						
VII	146	♂	35 42 46 50 56 60 69 69 75	1 94240 1 95211 1 24540 1 40509 1 30230 1 49111 0 99831 0 59559 1 05659	VIII	145	♀	30 35 47 59 67 70 78 80 86	1 85366 3 20681 3 72996 2 53249 1 22113 1 08584 0 95380 0 69743 0 92024
VII	147	♀	30 37 45 48 53 60 66 70 74	2 25060 2 89621 1 85917 1 16879 1 59389 1 56689 1 10110 0 81723 0 68080	VIII	160	♂	46 53 64 75 88 86 86 90 107 102	2 19094 2 35888 2 47355 2 27470 0 97758 0 16421 0 32474 1 56060 0 89401 0 13227

TABLE II—concl'd

Group	Rat number	Sex	Weekly weight (g)	Relative growth rate per cent per day	Group	Rat number	Sex	Weekly weight (g)	Relative growth rate per cent per day
VIII	161	♀	53	2 56391	IX	143	♀	36	4 40135
			63	1 45783				50	3 52871
			65	2 22296				59	2 70314
			86	2 78546				73	2 17494
			96	0 55894				80	1 33484
			93	-0 86927				88	0 68080
			85	-0 39473				88	0 31751
			88	1 84166				92	0 54671
			110	1 19324				95	0 15363
			104	0 08524				94	0 71480
			109	1 28947					
			120	0 95406					
VIII	162	♂	49	2 71981	IX	163	♂	67	3 42554
			60	1 90761				84	2 10800
			64	0 89404				90	1 24530
			68	1 77027				100	1 43337
			82	1 84166				110	0 80949
			88	0 34021				112	0 56174
			86	0 31751				119	1 38684
			92	1 00551					
			99	0 52380					
			99	0 62153					

The period of observation being too small no calculations have been made for groups IV and VI

The analysis of variance tables together with remarks regarding the applicability or otherwise of the logistic hypothesis are given below (*vide* Table III)

A comparison of growth curve of each experimental rat with a rat of corresponding sex put on basic diet has also been made. For this purpose the significance of the difference between the constants 'a' and 'b' of the straight lines fitted to the relative growth rates of the treated rats following the logistic hypothesis and the corresponding constants for basic diet control has been determined. The results have been included in the remarks column in Table III

TABLE III

Analysis of variance of relative growth rates of rats put on different treatments

Group number	Rat number	Sex	Source of variation	Sum of squares	D.F.	Variance	t	5 per cent point for t	REMARKS
I	160	σ	Linear regression	0.83083	1	0.83083	4.43	5.99	The logistic hypothesis is untenable. The growth is well represented by a straight line and is lower than that of rat No 163
			Deviation from linear regression	1.13427	6	0.18905			
	161	ϕ	Linear regression	1.60567	1	1.60567	10.29	5.59	The logistic hypothesis is untenable. The growth is well represented by a straight line and is lower than that of rat No 143
			Deviation from linear regression	0.61524	7	0.08789			
			Deviation from parabolic regression	0.26209	6	0.03308			
II	140	σ	Difference	0.35315	1	0.35315	8.08	5.99	The logistic hypothesis is not tenable but it must be borne in mind that the period of observation is rather short, and F value is not much below the 5 per cent point
			Linear regression	0.91993	1	0.91993	8.30	10.13	
	141	σ	Deviation from linear regression	0.33242	3	0.11081			The logistic hypothesis is untenable. The growth curve does not significantly differ from that of rat No 163
			Linear regression	0.54221	1	0.54221	57.13	6.61	
			Deviation from linear regression	0.04747	5	0.00949			
		σ	Deviation from parabolic regression	0.04694	4	0.01173			
			Difference	0.00053	0.1	0.000			

TABLE III—*contd.*

Group number	Rat number	Sex	Source of variation	Sum of square	D F	Variance	F	5 per cent point for F	REMARKS
III	156	♂	Linear regression	7 99641	1	7 99641	15 63	5 32	The logistic hypothesis is tenable The growth curve does not significantly differ from that of rat No 163
			Deviation from linear regression	4 09220	8	0 51153			
			Deviation from parabolic regression	4 08385	7	0 58341			
			Difference	0 00835	1	0 00835			
	157	♀	Linear regression	4 78043	1	4 78043	6 69	5 32	The logistic hypothesis is tenable The curve growth differs significantly from that of rat No 143
			Deviation from linear regression	5 71280	8	0 71410			
			Deviation from parabolic regression	5 10096	7	0 73728			
			Difference	0 55184	1	0 55184			
	158	♂	Linear regression	8 66020	1	8 66020	258 70	5 12	The logistic hypothesis is tenable The growth curve does not significantly differ from that of rat No 15 or 163
			Deviation from linear regression	0 30132	9	0 03348			
			Deviation from parabolic regression	0 25696	8	0 03212			
			Difference	0 04436	1	0 04436			
	159	♀	Linear regression	8 16876	1	8 16876	21 18	5 32	The logistic hypothesis is tenable The curve of growth is not significantly different from that of rat No 143 but different from that of rat No 167
			Deviation from linear regression	3 08557	8	0 38569			
			Deviation from parabolic regression	2 64361	7	0 37766			
			Difference	0 44196	1	0 44196			

IV {		σ	ϕ							Period of observation is too short	
165											
166											
169		{ ϕ	{ ϕ	Linear regression	2 48011	1	2 48011	8 32	5 50	{ The logistic hypothesis is not tenable	
				Deviation from linear regression	2 08052	7	0 20807				
				Deviation from parabolic regression	0 90938	6	0 15150				
				Difference	1 17711	1	1 17714	7 77	5 90		
170		{ σ	{ σ	Linear regression	1 20036	1	1 20036	3 28	5 50	{ The logistic hypothesis is not tenable	
				Deviation from linear regression	2 55072	7	0 30525				
171		{ ϕ	{ ϕ	Linear regression	3 04312	1	3 04312	0 20	5 50	{ The logistic hypothesis is not disproved but the curve is significantly different from that of rat No 143	
				Deviation from linear regression	3 43497	7	0 40071				
				Deviation from parabolic regression.	2 88810	6	0 48135				
				Difference	0 54087	1	0 54087				
168		{ ϕ	{ ϕ							{ Period of observation is too short	
VI											

The logistic hypothesis is not tenable

The logistic hypothesis is not tenable

The logistic hypothesis is not disproved but the curve is significantly different from that of rat No 143

Period of observation is too short

V

VI

TABLE III—*contd.*

Group number	Rat number	Sex	Source of variation	Sum of square	D F	Variance	F	5 per cent point for F	REMARKS
VII	146	♂	Linear regression	1 05280	1	1 05280	15 58	5 59	The hypothesis of logistic curve is tenable, but the curve of growth is significantly lower than that of rat No 163. Further, the growth is better depicted by a straight line than a logistic curve
			Deviation from linear regression	0 47252	7	0 06750			
			Deviation from parabolic regression	0 45119	6	0 07520			
			Difference	0 02133	1	0 02133			
	147	♀	Linear regression	3 06426	1	3 06420	21 98	5 59	The hypothesis of logistic curve is tenable, but the curve of growth is significantly lower than that of rat No 143. Further, the growth is better depicted by a straight line than a logistic curve
			Deviation from linear regression	0 97592	7	0 13942			
			Deviation from parabolic regression	0 97590	6	0 16265			
			Difference	0 00002	1	0 00002			
144		♂	Linear regression	6 44863	1	6 44863	20 05	5 59	The hypothesis of logistic curve is tenable. The logistic curve of growth is a little lower than that of rat No 163
			Deviation from linear regression	2 25180	7	0 32169			
			Deviation from parabolic regression	2 23031	6	0 37172			
			Difference	0 02149	1	0 02149			

145	♀	Linear regression Deviation from linear regression Deviation from parabolic regression Difference	5 55878 4 23542 1 03114 1 20218	1 7 0 1	5 55878 0 60504 0 50552 1 20218	9 10 5 59	The hypothesis of logistic curve is tenable. But the logistic curve is significantly different from that of rat No 143. The growth during the experimental period is less, but towards the end of the experiment the growth is relatively more.
100	♂	Linear regression Deviation from linear regression Deviation from parabolic regression Difference	4 80030 3 37033 3 37590 0 00037	1 8 7 1	4 80030 0 42304 0 48228 0 00037	11 30 5 32	The hypothesis of logistic curve is tenable. The logistic curve of growth is not significantly different from that of rat No 144, but is a little lower than that of rat No 103.
101	♀	Linear regression Deviation from linear regression	2 82000 11 75892	1 10	2 82000 1 17580	2 41 4 00	The hypothesis of logistic curve is not tenable.
102	♂	Linear regression Deviation from linear regression Deviation from parabolic regression Difference	3 71000 2 27788 2 07810 0 10978	1 8 7 1	3 71000 0 28474 0 20087 0 10978	13 03 5 32	The hypothesis of logistic curve is tenable. The logistic curve of growth is not significantly different from that of rats Nos 144 and 100, but a little lower than that of rat No 102.

TABLE III—*concd*

Group number	Rat number	Sex	Source of variation	Sum of square	D F	Variance	F	5 per cent point for F	REMARKS
IX	143	♀	Linear regression	19 12364	1	19 12364	301 87	5 32	The hypothesis of logistio curve is tenable as the linear regression function is significant, and the reduction when a parabolio is fitted is not significant
			Deviation from linear regression	0 50878	8	0 06335			
			Deviation from parabolic regression	0 50397	7	0 07197			
			Difference	0 00281	1	0 00281			
	163	♂	Linear regression	4 02661	1	4 02661	13 81	6 61	The hypothesis of logistio curve is tenable as the linear regression function is significant, and the reduction when a parabolio is fitted is not significant
			Deviation from linear regression	1 45836	5	0 29167			
			Deviation from parabolic regression	0 50617	4	0 12654			
			Difference	0 95219	1	0 95219			

INVESTIGATIONS INTO THE EPIDEMIOLOGY OF EPIDEMIC DROPSY

Part XIV

FEEDING EXPERIMENTS ON HUMAN SUBJECTS TO TEST THE TOXICITY OF SOME OF THE DERIVATIVES AND MODIFICATIONS OF ARGEMONE OIL

BY

R B LAL, MB, BS, DPH, DTM & H, DB, FNI,
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AND

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*(An Inquiry under the Indian Research Fund Association)
(From the Department of Epidemiology and Vital Statistics, All-India
Institute of Hygiene & Public Health, Calcutta)*

[Received for publication, May 22, 1941]

IN the last communication of this series (Lal *et al* , 1941) it was shown that both the crystalline free base (c f b) and the white crystalline substance (w c s) derived from argemone oil were biologically active. They were capable of inducing on rats the essential histological changes of the disease of man and the former also of causing definite retardation in their growth curve. It was shown that argemone oil from which the c f b has been extracted or which has been exposed to light for sufficiently long time became biologically inactive. A parallelism between the chemical and the biological tests was thus satisfactorily demonstrated. However, before these results could be applied to human beings, it was necessary to carry out experiments on man.

Three human feeding experiments were carried out. The general experimental technique reported in Part V of this series (*vide* Lal and Roy, 1937) was adopted.

EXPERIMENT I

The object of the experiment was to test the toxic properties of c f b. The basic food was the same as in the original experiment referred to above, but the rice, the *chapattis* and other articles of food except culinary fat were supplied from general kitchen. Besides, the early morning meal was taken by the volunteers in the general file and the special oil was used for cooking two meals only. Considerable difficulty was experienced in obtaining healthy volunteers in sufficient numbers and this restricted the numbers included in the various groups.

Group 1—Consisted of 3 volunteers to begin with. Mustard oil containing c f b equivalent to 5 per cent argemone oil according to colorimetric test was the culinary fat. One volunteer refused co-operation on the second day and his place was taken by another man on the fourth day.

Group 2—Two volunteers were included in this group one of whom joined 4 days after the commencement of the experiment. Their food was cooked in 5 per cent argemone oil from which c f b had been removed according to the technique described in Part XII (*vide* Mukherji *et al*, 1941).

Group 3—Consisting of 3 volunteers, was fed on 5 per cent argemone oil to serve as positive control. This part of the experiment had to be abandoned after 5 days for want of co-operation.

The first two groups continued on the special diet for 20 days. The details of feeding and the results obtained are stated in Table I—

TABLE I.

Experiment I Details of feeding and results

Group number	Individual number	Period of feeding (days)	Amount of oil taken (ounces)	RESULTS
1	D	17	28½	No symptoms
	E	19	40	Fever for one day with pain in left flank after taking 8½ ounces of oil
	F	20	43	No symptoms
2	G	20	41½	Slight fever with cold and headache for one day after taking 27½ ounces of oil
	H	17	38	No symptoms

TABLE I—*concl'd*

Group number	Individual number	Period of feeding (days)	Amount of oil taken (ounces)	RESULTS
1	A	1	2	Withdraw co operation after one day
	B	4	9	Fever for 2 days with discomfort in abdomen after taking 9 ounces of oil He then withdrew co operation
	C	4	8½	Withdraw co operation

EXPERIMENT II

Object—To test the toxicity of c f b, w c s and light-treated argemone oil

The experimental technique was the same as in the previous experiment except that the diet was more liberal. Thus, mutton was substituted for fish on alternate days instead of once a week and a piece of lemon twice a day instead of tomato and during the first 11 days, 4 ounces of milk curd (*dahi*) was given on alternate days. Later on it was replaced by 8 ounces of milk. To begin with, the oil was given in smaller quantities (1½ ounces per head per day) but from the tenth day it was increased to 2 ounces per head per day. There were six groups of 3 volunteers each.

Group 1—Received 5 per cent mixture of light-treated argemone oil in pure mustard oil. The method of preparation has been described in Part IX of this series (*vide* Lal *et al*, 1940). The resultant mixture gave extinction coefficient value of 0.48 at 4,600 Å U which was equivalent to 0.45 per cent argemone oil.

Group 2—The oil was the same as in group 1 with the addition of c f b equivalent to 5 per cent argemone oil.

Group 3—Received w c s in pure mustard oil equivalent to 5 per cent argemone oil.

Group 4—Was put on 5 per cent argemone oil in pure mustard oil from which c f b have been removed. The extinction coefficient value of the resultant mixture was 0.1 at 4,600 Å U which was roughly equivalent to the same per cent of argemone oil.

Group 5—Received the same oil as group 4 with the addition of c f b equivalent to 5 per cent argemone oil, the mixture being incubated at 37°C overnight. The object of including this group was to see whether in removing c f b a radicle was left behind which though quite non-toxic by itself would re combine with c f b to form the toxic substance, assuming c f b by itself to be non-toxic.

Group 6—This was positive control with 5 per cent argemone oil. The details of feeding and of the results obtained are given in the *Appendix*.

Three human feeding experiments were carried out. The general experimental technique reported in Part V of this series (*vide* Lal and Roy, 1937) was adopted.

EXPERIMENT I

The object of the experiment was to test the toxic properties of c f b. The basic food was the same as in the original experiment referred to above, but the rice, the *chapattis* and other articles of food except culinary fat were supplied from general kitchen. Besides, the early morning meal was taken by the volunteers in the general file and the special oil was used for cooking two meals only. Considerable difficulty was experienced in obtaining healthy volunteers in sufficient numbers and this restricted the numbers included in the various groups.

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Group 2—Two volunteers were included in this group one of whom joined 4 days after the commencement of the experiment. Their food was cooked in 5 per cent argemone oil from which c f b had been removed according to the technique described in Part XII (*vide* Mukherji *et al*, 1941).

Group 3—Consisting of 3 volunteers, was fed on 5 per cent argemone oil to serve as positive control. This part of the experiment had to be abandoned after 5 days for want of co-operation.

The first two groups continued on the special diet for 20 days. The details of feeding and the results obtained are stated in Table I—

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	F	20	43	No symptoms
2	G	20	41½	Slight fever with cold and headache for one day after taking 27½ ounces of oil
	H	17	38	No symptoms

effect re-combination into the original toxic substance or loss of some constituent which might have occurred during the process of isolation of c f b may explain it

CONCLUSIONS

1 Fractions obtained from argemone oil by the processes of saponification and extraction with hydrochloric acid gas have not proved toxic to man although they react specifically to chemical tests and induce essential histological changes in white rats

2 The residue of argemone oil left after extraction of the fraction with hydrochloric acid gas is non-toxic to man

3 Adequate exposure of argemone oil to light renders it non-toxic

4 These observations suggest that the treatments so far employed, isolate only a portion of the toxic radicle and neither of the split products are toxic. Re-combination of the split products into the original toxic molecule has not been effected by the methods so far employed

ACKNOWLEDGMENTS

It is a great pleasure to record our gratitude to Lieut-Colonel M. A. Singh, I M S, and Major S. Annaswami, I M S, for their whole-hearted co-operation in conducting the experiments. We are also thankful to Lieut-Colonel E. W. O'G. Kirwan, C I E, I M S, and Dr H. D. Banerjee, for the trouble they took in carrying out ophthalmic examination of the volunteers. We are also grateful to Dr J. B. Grant, for his visits to the institution for inspecting the experiments.

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EXPERIMENT III

This was virtually a continuation of experiment II and consisted of only one group of 3 volunteers. The oil used was a 5 per cent mixture of light-treated argemone oil in pure mustard oil to which the w c s equivalent to 5 per cent argemone oil had been added. The feeding was carried out for 23 days and each volunteer consumed 45 ounces of oil.

The results are given in Table II —

TABLE II

Experiment III — Details of feeding and results

Group number	Individual number	Period of feeding (days)	Amount of oil consumed (ounces)	RESULTS
1	I	23	45	No symptoms
	II	23	45	Loose motions with discomfort in the abdomen and pain all over the body after the consumption of 32 ounces of oil. It continued for 3 days.
	III	23	45	Pain in the lower limbs, after the consumption of 45 ounces of oil.

DISCUSSION

The results obtained in experiment II, group 6, confirmed the conclusion regarding the causative rôle of *Argemone mexicana* oil in epidemic dropsy. Although some suspicious signs and symptoms were produced by w c s and c f b fractions of argemone oil, they were not sufficiently grave to justify being labelled as epidemic dropsy. In this respect the chemical and the biological tests failed as complete tests of toxicity. Since, however, these purified fractions are not likely to be met with in oil as sold in the market, the practical value of either tests does not suffer in any way. Since both the isolated fractions and the residue left after extraction of c f b are non-toxic, the simple inference that may be drawn is that the latter constitutes only a part of the toxic molecule found in argemone oil. Since, however, addition of c f b or w c s to the residue left after the extraction of the former fraction does not result in a toxic product, it may be argued that non-toxicity of the residue or of the fractions is due to modification of the toxic substance rather than to its being split up. While, we are not in a position to dispute this possibility, the separation of c f b by a relatively gentle treatment would support the hypothesis of splitting rather than of radical modification, especially when it is remembered that the toxic substance in the oil is able to withstand heating to the high temperatures involved in cooking food. The simple process of mixing the c f b or w c s. with the residue may lack the necessary chemical energy to

Group 1 (argemone oil modified by light), volunteer No 6—Well developed, healthy and bright, cane worker, 27 years, been in the institution for 3 months, no previous history of illness, no complaints since admission Height 5 feet $1\frac{3}{4}$ inches Weight 115 lb

Duration of feeding 1st August, 1940 to 30th August, 1940

Total amount of oil consumed 59 ounces.

Signs and symptoms after the commencement of feeding Nil.

General appearance Unaltered

Weights 28th July, 1940, 115 lb , 7th August, 1940, 118 lb , 17th August, 1940, 120 lb , 30th August, 1940, 120 lb , 28th September, 1940, 120 lb

Urine Nothing abnormal

Group 2 (light treated argemone oil with c f b added), volunteer No 7—Well developed, bright and active, durrie weaver, 34 years, been in the institute for 16 months, no history of previous illness excepting a halo on the right side and occasional glossitis Height 5 feet $6\frac{1}{2}$ inches Weight 135 lb

Duration of feeding 1st August, 1940 to 30th August, 1940

Total amount of oil consumed 58 ounces

Signs and symptoms after the commencement of feeding —

Subjective symptoms—

19th August, 1940 Discomfort in the abdomen after the consumption of $34\frac{1}{2}$ ounces of oil

Physical signs—nothing abnormal.

Weights 28th July, 1940, 135 lb , 7th August, 1940, 135 lb , 17th August, 1940, 135 lb , 30th August, 1940, 136 lb , 28th September, 1940, 133 lb

Urine Nothing abnormal

Group 2 (light treated argemone oil with c f b added), volunteer No 8—Well developed, healthy and active, durrie weaver, 24 years, been in the institution for one year, no history of previous illness, no complaints since admission Height 5 feet 3 inches Weight 128 lb

Duration of feeding 14th August, 1940 to 30th August, 1940

Total amount of oil consumed 35 ounces

Signs and symptoms after commencement of feeding —

Subjective symptoms—

19th August, 1940 Pain and discomfort in the abdomen after the consumption of $11\frac{1}{2}$ ounces of oil.

Physical signs Nil

Weights 4th August, 1940, 128 lb , 17th August, 1940, 125 lb , 30th August, 1940, 131 lb , 28th September, 1940, 129 lb

Urine Nothing abnormal

Group 2 (light-treated argemone oil with c f b added), volunteer No 9—Well developed, active and bright, coir string maker, 35 years, been in the institution for 4 months, no history of previous illness and no complaints since admission Height 5 feet 5 inches Weight 119 lb

Duration of feeding 1st August, 1940 to 30th August, 1940

Total amount of oil consumed 58 ounces.

Signs and symptoms after commencement of feeding Nil

Weights 28th July, 1940, 119 lb , 7th August, 1940, 117 lb , 17th August, 1940, 117 lb , 30th August, 1940, 119 lb , 28th September, 1940, 121 lb

Urine Nothing abnormal.

Group 3 (w c s in mustard oil), volunteer No 10—Well developed, active and bright, brush maker, 24 years, been in the institution for 2 months, no previous history of illness No complaints since admission excepting a peritonaxillar abscess Height 5 feet $4\frac{1}{2}$ inches Weight 139 lb

Duration of feeding 1st August, 1940 to 30th August, 1940

APPENDIX.

Examination of the individual volunteers

For the sake of brevity, the negative findings have been omitted and mention is made only of such signs and symptoms as have a direct bearing on the disease

EXPERIMENT II

Group 1 (argemone oil modified by light), volunteer No 4—Well developed, active and bright, 25 years, durrie weaver, no previous illness, no complaints since admission 2 months ago Height 5 feet 7 inches Weight 140 lb

Duration of feeding 1st August, 1940 to 27th August, 1940

Total amount of oil consumed 52½ ounces

Signs and symptoms after the commencement of feeding —

Subjective symptoms—

19th August, 1940 Loose motions after consumption of 34 ounces of oil

20th August, 1940 Gurgling sensation in the abdomen

26th August, 1940 Pain around the umbilicus

27th August, 1940 Pain, burning sensation all over the body and fever during the previous night

28th September, 1940 Weakness and pain over the precordia

Physical signs—

General appearance—not much changed

Weights 28th July, 1940, 138 lb , 7th August, 1940, 135 lb , 17th August, 1940, 137 lb , 30th August, 1940, 134 lb , 28th September, 1940, 139 lb

28th August, 1940 Slight rise of temperature (99.6°F) which persisted for 3 days and then subsided

5th September, 1940 Heart examined—nothing abnormal found no œdema of legs noticed, nor pitting on pressure

Urine Nothing abnormal

Group 1 (argemone oil modified by light), volunteer No 5—Well developed, active and bright, 23 years, durrie weaver, been in the institution for one year, no previous illness, no complaints since admission Height 5 feet 4 inches Weight 126 lb

Duration of feeding 1st August, 1940 to 20th August, 1940

Total amount of oil consumed 37 ounces

Signs and symptoms after the commencement of feeding —

Subjective symptoms—

20th August, 1940 Fever with pain all over the body and perspiration after consumption of 36½ ounces of oil

24th August, 1940 A sensation of numbness on the right side of the face and arm

26th August, 1940 Numbness and pain all over the body

5th September, 1940 Distaste for food

Physical signs—

General appearances Weak and slightly reduced

Weights 28th July, 1940, 126 lb , 7th August, 1940, 125 lb , 17th August, 1940, 125 lb , 30th August, 1940, 122 lb , 28th September, 1940, 120 lb

20th August, 1940 A high rise of temperature (103°F) with perspiration The temperature came down to normal, the very next day and the physical pain also diminished

Examined for œdema up to 28th September, 1940, but nothing found

Urine Nothing abnormal

Group 5 (argemone oil minus cfb), volunteer No 14—Well developed, active and bright, durrie weaver, 35 years, been in the institution for one year, history of meningitis 3 years back and gonorrhoea about the same time, complained of occasional pain over the splenic region with fever
Height 5 feet 1 inch Weight 117 lb

Duration of feeding 1st August, 1940 to 22nd August, 1940 (refused co operation)

Total amount of oil consumed 41 ounces

Signs and symptoms after the commencement of feeding Nil

Weights 28th July, 1940, 117 lb 7th August, 1940, 114 lb , 17th August, 1940, 120 lb ,
30th August, 1940, 122 lb , 28th September, 1940, 110 lb

Urine Nothing abnormal

Group 4 (argemone oil minus cfb) volunteer No 15—Well developed, healthy and bright, carpenter, 27 years, been in institution for 2 months, history of hookworm, complained of occasional hyperacidity with pain over gastric region Height 5 feet 7½ inches Weight 144 lb

Duration of feeding 1st August, 1940 to 30th August, 1940

Total amount of oil consumed 57½ ounces

Signs and symptoms after the commencement of feeding Nil

Weights 28th July, 1940, 144 lb , 7th August, 1940, 142 lb 17th August, 1940, 142 lb ,
30th August, 1940, 144 lb , 28th September, 1940, 140 lb

Urine Nothing abnormal

Group 5 (argemone oil minus cfb plus cfb), volunteer No 16—Moderately developed, active and bright, coir mat maker, 28 years, history of syphilis 6 years back, hard inguinal glands felt, no complaints since admission to the institution one month ago Height 5 feet 4½ inches Weight 110 lb

Duration of feeding 1st August, 1940 to 25th August, 1940 (refused meal after this date)

Total amount of oil consumed 43 ounces

Signs and symptoms after the commencement of feeding —

Subjective symptoms—

24th August, 1940 Haematuria after the consumption of 42½ ounces of oil

19th September, 1940 Diarrhoea with blood and mucus in the stool.

Physical signs—

General appearance Not altered

Weights 28th July, 1940, 110 lb , 7th August, 1940, 108 lb , 17th August, 1940, 110 lb ,
30th August, 1940, 112 lb

25th August, 1940 Kept on observation in the hospital but no trace of blood in urine could be found out

Group 5 (argemone oil minus cfb plus cfb), volunteer No 17—Well developed, active and bright, durrie weaver, 28 years, no history of past illness, no complaints since admission to the institution 5 years ago excepting occasional glossitis. Height 5 feet 4 inches Weight 134 lb

Duration of feeding 10th August, 1940 to 30th August, 1940

Total amount of oil consumed 43 ounces

Signs and symptoms after the commencement of feeding —

Subjective symptoms—

21st August, 1940 Pain in the joints after the consumption of 23 ounces of oil.

Physical signs Nil

Weights 10th August, 1940, 134 lb , 17th August, 1940, 136 lb , 30th August, 1940,
139 lb , 28th September, 1940, 139 lb

Urine Nothing abnormal.

Group 5 (argemone oil minus cfb plus cfb), volunteer No 18—Sparely built but active and bright, coir string worker, 34 years, been in the institution for 4 months, history of ankylostomiasis and

Total amount of oil consumed 58 ounces

Signs and symptoms after the commencement of feeding *Nil*

Weights 21st July, 1940, 139 lb , 7th August, 1940, 135 lb , 17th August, 1940, 136 lb ,
30th August, 1940, 141 lb , 28th September, 1940, 138 lb

Urine Nothing abnormal

Group 3 (w c s in mustard oil), volunteer No 11—Moderately developed, active and bright, brush maker, 26 years, been in the institution for 2 months, no history of previous illness, no complaints since admission Height 5 feet 2½ inches Weight 124 lb

Duration of feeding 1st August, 1940 to 30th August, 1940

Total amount of oil consumed 58 ounces

Signs and symptoms after the commencement of feeding —

Subjective symptoms—

13th August, 1940 Pain all over the body after consumption of 21 ounces of oil

20th August, 1940 Weakness, physical pain and dimness of vision

22nd August, 1940 Pain around the umbilicus He continued complaining of the above symptoms up to the end of the experiment

General appearance Not altered

Weights 21st July, 1940, 124 lb , 7th August, 1940, 119 lb , 17th August, 1940, 120 lb
30th August, 1940, 122 lb , 28th September, 1940, 120 lb

22nd August, 1940 Eye examined No abnormality found He was examined repeatedly for œdema of legs up to 22nd October, 1940, but no œdema was found

Group 3 (w c s in mustard oil), volunteer No 12—Well developed, active and bright, brush maker, 32 years, been in the institution for one year, no previous illness excepting occasional dysentery and glossitis Height 5 feet 3 inches Weight 140 lb

Duration of feeding 1st August, 1940 to 30th August, 1940

Total amount of oil consumed 55½ ounces

Signs and symptoms after the commencement of feeding —

Subjective symptoms—

13th August, 1940 Pain all over the body after consumption of 21 ounces of oil

20th August, 1940 Weakness and dimness of vision

27th August, 1940 Giddiness and pain all over the body He continued complaining of these symptoms up to the end of the experiment

Physical signs—

General appearance Not much altered

Weights 21st July, 1940, 140 lb , 7th August, 1940, 139 lb , 17th August, 1940, 139 lb ,
30th August, 1940, 142 lb , 28th September, 1940, 140 lb

Eye examined by ophthalmologist on 22nd August, 1940, 29th August, 1940 and 23rd September, 1940

Short sightedness R E—6/9, L E—6/12 No other abnormality He was examined repeatedly for œdema of legs but none found

Group 4 (argemone oil minus c f b), volunteer No 13—Well developed, active and bright, tape weaver, 28 years, been in the institution for 2 months, no history of previous illness, no complaint since admission excepting occasional hyperacidity of the stomach Height 5 feet 5½ inches Weight 126 lb

Duration of feeding 1st August, 1940 to 30th August, 1940

Total amount of oil consumed 52 ounces

Signs and symptoms after commencement of feeding *Nil*

Weights 28th July, 1940, 126 lb , 7th August, 1940, 123 lb , 17th August, 1940, 123 lb ,
30th August, 1940, 125 lb , 30th September, 1940, 120 lb

Urine Nothing abnormal

Weights 28th July, 1940, 142 lb , 7th August, 1940, 142 lb , 17th August, 1940, 142 lb
28th September, 1940, 139 lb

22nd August, 1940 Eye examined by ophthalmologist R E 6/9 Otherwise normal

19th August, 1940 Doubtful œdema of legs

21st August, 1940 Definite œdema, pitting on pressure commenced after consumption of 38 ounces of oil The œdema gradually increased and on 24th August, 1940, it became very much marked and exhibited characteristic red flush

3rd September, 1940 Heart examined, nothing abnormal found excepting marked accentuation of pulmonary 2nd sound with slight tachycardia Pulse 90

11th September, 1940 Marked pigmentation of face

Urine Nothing abnormal

Group 6 (argemone oil), volunteer No 3—Well developed, active, weaver, 32 years, no previous illness excepting occasional enlargement of left testis, no complaints since admission 1½ years ago excepting occasional glossitis Height 5 feet 7 inches Weight 139 lb

Duration of feeding 1st August, 1940 to 30th August, 1940

Total amount of oil consumed 43 ounces

Signs and symptoms after commencement of feeding —

Subjective symptoms—

12th August, 1940 Pain in joints after consumption of 19 ounces of oil

20th August, 1940 Weakness and dimness of vision and loss of appetite

21st August, 1940 Pain and discomfort in the abdomen and incomplete evacuation of bowels

10th September, 1940 Fever at night and weakness

Physical signs—

General appearance run down and much reduced

Weights 28th July, 1940, 139 lb , 7th August 1940, 135 lb , 17th August, 1940, 135 lb ,
30th August, 1940, 136 lb , 29th September, 1940, 129 lb

22nd August, 1940 Eyes examined by ophthalmologist No abnormality found

27th August, 1940 Definite œdema of legs, pitting on pressure after consumption of 40 ounces of oil

11th September, 1940 Heart pulmonary 2nd sound accentuated Pulse 82

Urine Nothing abnormal

bubo left side with some hard glands palpable in inguinal region Complained of occasional pain around the umbilicus Height 5 feet 4 inches Weight 112 lb

Duration of feeding 1st August, 1940 to 30th August, 1940 (missed one meal for pain in the abdomen)

Total amount of oil consumed 42 ounces

Signs and symptoms after the commencement of feeding —

Subjective symptoms—

14th August, 1940 Pain in the abdomen after the consumption of 21 ounces of oil

19th August, 1940 Pain in the abdomen and precordia and refused meal in the evening

19th September, 1940 Perennial abscess

Physical signs—

General appearance not much altered but after the abscess he was much reduced

Weights 28th July, 1940, 112 lb, 7th August, 1940, 109 lb, 17th August, 1940, 109 lb, 30th August, 1940, 110 lb, 28th September, 1940, 101 lb

19th September, 1940 Perennial abscess and was admitted into the hospital

Urine Nothing abnormal

Group 6 (argemone oil), volunteer No 1 —Well developed, active and bright young man, 26 years history of typhoid and bube 4½ years back, no complaints since admission to the institution 14 months ago excepting occasional pain around the umbilicus A few enlarged glands palpable in the inguinal region Height 5 feet 6 inches Weight 137 lb

Duration of feeding 1st August, 1940 to 30th August, 1940

Total amount of oil consumed 49 ounces

Signs and symptoms after commencement of feeding —

Objective symptoms—

12th August, 1940 Pain in the joints after the consumption of 19 ounces of oil

20th August, 1940 Weakness and dimness of vision

22nd August, 1940 Loose motions and loss of appetite

11th September, 1940 Sleeplessness and weakness

Physical signs—

General appearance dull, seedy and much reduced

Weights 28th July, 1940, 137 lb, 7th August, 1940, 137 lb, 17th August, 1940, 133 lb, 30th August, 1940, 134 lb, 28th September, 1940, 129 lb

22nd August, 1940 Eye examined by ophthalmologist No abnormality found

20th August, 1940 Definite œdema of both the legs, pits on pressure and increased in the evening began after consumption of 47 ounces of oil

Urine No abnormality found

Group 6 (argemone oil), volunteer No 2 —Well developed, active and bright, weaver, 33 years, no previous illness excepting gonorrhœa, no complaint since admission 2 years ago excepting occasional glossitis Height 5 feet 5 inches Weight 142 lb

Duration of feeding 1st August, 1940 to 25th August, 1940

Total amount of oil consumed 45 ounces

Signs and symptoms after commencement of feeding —

Subjective symptoms—

12th August, 1940 Pain in the joints after the consumption of 19 ounces of oil

13th August, 1940 Loss of appetite

20th August, 1940 Pain all over the body, weakness and dimness of vision

23rd August, 1940 Œdema of legs, palpitation and dyspnoea on exertion

25th August, 1940 Pain in the abdomen

27th August, 1940 Pain over precordia

Physical signs—

General appearance dull and much reduced.

FOOD AND FEEDING HABITS OF THE GLASSFISHES
(*AMBASSIS* CUV & VAL) AND THEIR
BEARING ON THE BIOLOGICAL
CONTROL OF GUINEA-WORM
AND MALARIA *

BY

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INTRODUCTION

THE glassfishes, so called owing to their translucent, fragile body, comprise some of the smallest perches and belong to the genus *Ambassis* Cuvier and Valenciennes (family Centropomidae†). While the majority are marine, a few species have become acclimatized to fresh-water conditions, and of these about half a dozen are found all over India and Burma. *Ambassis nama* (Ham) and *A. rangi* (Ham) are the most widely distributed fresh-water species in India, occurring extensively both in standing and in running waters.

The mosquitocidal propensities of *Ambassis*, mainly judged from aquarium experiments, have attracted the attention of various writers. While most (Chaudhuri, 1911, Fry, 1912, O'Donnell, 1912, Murphy, 1914, D'Abreu, 1925) of these workers considered them as efficient mosquitocides, Southwell (1920) held that they are 'species of less importance' in mosquito control than *Aplocheilichthys*. Prasad and Hora (1936) noted that the species of *Ambassis* 'are rather delicate fishes, and can, therefore, be used with advantage only in the neighbourhood of their normal habitats'.

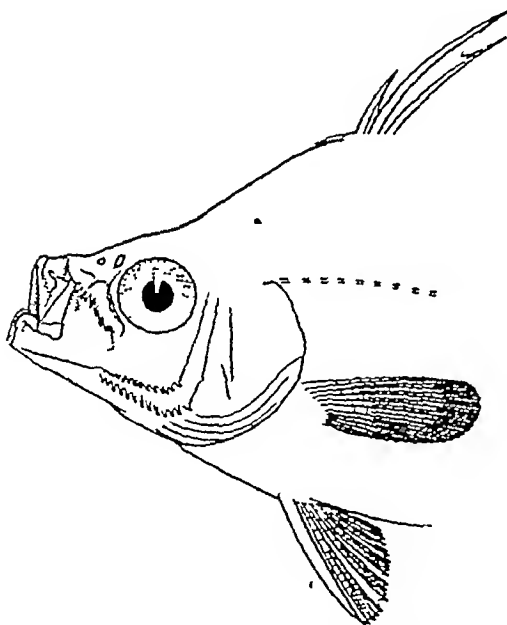
Cyclopsicidal tendencies were observed in the year 1937 in one species, *A. gymnocephalus* (Lacépède), collected from the Fort St George moat, Madras,

* Presented as part of a thesis for the degree of Doctor of Science of the University of Madras.

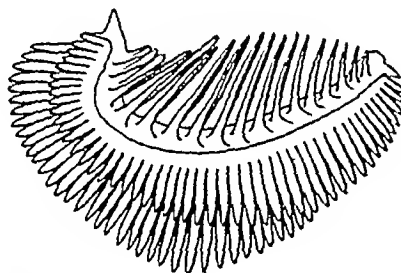
† The classification suggested by Weber and de Beaufort (1929) is followed in this work. In the systematic arrangement of the perches (Job, 1940a) the family name was accidentally omitted.

have been taken in accidentally. From the nature of their food it appears that these fishes feed at all layers, but subsist mainly on the minute Entomostracans which flourish in mid-water. The protrusible mouth with upturned gape possessed by these species (Text-figure 1) is presumably used for taking in large gulps of water. An examination of the gill arches showed that the combs of horny, setiform prolongations from the anterior gill arches (Text-figure 2) form a very efficient sieve-like apparatus for the collection of enormous numbers of such microcrustaceans as Cyclops. Sometimes, however, the fish resort to sight feeding as well, as is evident from their gut-contents, the mosquito larvæ were probably captured from near the surface and the worms picked up from the bottom.

FEEDING MECHANISM OF *Ambassis ranga* (HAMILTON)



Text figure 1 Anterior part of the fish showing the protrusible mouth $\times ca\ 3$



Text-figure 2 Anterior view of the first right gill showing the setiform gill rakers $\times ca\ 8\frac{1}{2}$

FIELD* EXPERIMENTS

To determine the effect of the introduction of *A. nama* and *A. ranga* on the population of mosquito larvæ and Cyclops in their natural habitats, three central chambers termed hereunder as *a*, *b* and *c* respectively, each 8 feet by 6 feet, in the borrowpits situated between telegraph posts 19/4 and 19/5 up, at Fuleshwar along the Bengal Nagpur Railway, were chosen. The chambers were in the middle

in the course of the writer's investigations (*vide* Job, 1940a) on the nutrition of perches, and it was stated that 'The cyclopscidal tendencies of the fish point to its probable utility in the biological control of guinea-worm disease. But sufficient data are not yet available for discussing the point'

While investigating the natural feeding habits of the glassfishes with a view to evaluating their relative utility in anti-mosquito measures, their more marked cyclopscidal tendencies became clear. Accordingly, the feeding habits of *A. nama* and *A. ranga* were studied under both natural and laboratory conditions, and the results are presented in this communication.

ANALYSES OF GUT-CONTENTS OF GLASSFISHES FROM NATURAL HABITATS

Two hundred and sixty-eight specimens of *A. nama* and three hundred and thirteen of *A. ranga* were collected on different dates during 1939 and 1940 from the ponds and pools around the Calcutta Corporation's Waterworks at Pulta in the 24-Parganas and from the borrowpits, tanks and canals in the malarious stations of Ulubaria and Fuleshwar in the Hooghly Delta Section of the Bengal Nagpur Railway. Examination of the pieces of water concerned revealed a fairly high mosquito larval incidence in many of them, while plankton collections often showed appreciable numbers of Cyclops and other Entomostracans in the microfauna.

The specimens of *A. nama* ranged from 19 mm to 66 mm in length. One hundred and thirty-one of them were males, one hundred and twenty females and the remainder immature. The specimens of *A. ranga* were from 14 mm to 57 mm in length, one hundred and sixty-four of them were males, one hundred and thirty-eight females and the rest immature. In many of the adults the gonads were well developed and full, while in a few they were in a spent condition.

All the fishes were captured alive. The gut-contents were studied as in the case of *Aplocheilichthys panchax* (*vide* Job, 1941b). Only in twenty-four specimens of *A. nama* and forty-one of *A. ranga* were the guts absolutely empty. These, however, were not confined either to ripe males and females or to spent individuals. Several specimens with fully ripe or spent gonads, on the other hand, had full stomachs, and therefore it is evident that these fishes do not abstain from feeding during the breeding season.

Both qualitative and quantitative analyses of the food were done and, besides volumetric determination of the constituent elements by Pearse's (1915) method, numerical proportions were also reckoned, since sometimes a single Oligochaete bit or other similar large-sized item far outsized hundreds of Copepods. The results of the food analyses of *A. nama* and *A. ranga* from natural habitats are detailed in the first columns of Tables I and II respectively.

It was found that Cyclops formed the major portion of the natural diet of both the species. The other common items were aquatic stages of mosquitoes and Ostracods, Daphnids and other lower crustaceans, while Chironomid larvae, bits of earthworms and packets of fish scales were sometimes met with. The amounts of vegetable matter and inorganic particles were practically negligible and might

The above field experiments confirmed beyond doubt that the glassfishes under consideration, which are not typical surface feeders, are helpful only to a small extent in the reduction of mosquito larvæ, and these mainly Culicines, while they are remarkably effective in the destruction of Cyclops, and that, comparatively speaking, *A. nama* is much more active than *A. ranga* as a larvivore, while the latter is much more efficient than the former as a cyclopscivore.

FEEDING EXPERIMENTS

Series I —

To study the feeding habits of the species under artificial conditions of an aquarium, in the presence of equal amounts of the different items of their natural diet, twelve specimens of each species were experimented with, on lines similar to those of the feeding experiments, series I, of *Aplocheilichthys panchax* (vide Job, 1941b). The food supplied consisted of tow-net collections of mid-water and surface plankton mainly composed of Entomostracans like Daphnids, Cyclops, Ostracods, etc., the young of Decapods, Water-boatmen and other Hemipterans, with spoonfuls of the aquatic stages of Anopheline and Culicine mosquitoes, larvæ of Chironomids and other insects and chopped up earthworms. While exact equalization of the items was difficult, they were more or less balanced with dips, and the rich varied menu thus obtained was introduced into prepared aquarium troughs in which the fishes had been starved for twelve hours. After three-quarters of an hour, the fishes were captured and their gut-contents studied. The results are shown in the second column of Tables I and II respectively. The relative amounts of mosquito larvæ, Chironomids and earthworm bits eaten were found to be greater in the feeding experiments than in fishes collected from their natural habitats. Evidently when kept starving in the limited environment of an aquarium, sight was used far in excess in feeding and the large conspicuous elements available in the water were swallowed. It was, however, seen that considerable numbers of Cyclops were also consumed. The amounts of the different stages of Anophelines and Culicines taken showed that the early instar larvæ formed the major part. A few late instars and still fewer pupæ were taken, while imagines were extremely rare. Between Anophelines and Culicines, more of the latter were consumed. Comparatively more mosquito larvæ were eaten by *A. nama*, while *A. ranga* consumed more Cyclops.

It may be inferred from the above experiments that when kept starving under artificial conditions of an aquarium, the glassfishes feed more by sight upon larger elements than by filtration on microfauna. Even in the aquarium *A. nama* is the better mosquitocide of the two, while *A. ranga* is the better cyclopscivore.

Series II —

The cyclopscivorous and mosquitocidal propensities of the glassfishes having been determined, further experiments were carried out to determine the maximum capacities of the fishes for consuming Cyclops and mosquito larvæ. Accordingly,

of the borrowpits, which had dried up by the 15th of December, 1940, leaving water to a depth of only 3 feet in the chambers. The ecological conditions of the chambers were more or less similar. The water contained small bits of the common weed, *Lagarosiphon rosburghii* Benth, patches of green algæ and planktonic microflora. The fauna consisted of a few young frogs, snails, larvæ of mosquitoes and other insects and several lower crustaceans including Cyclops. Before starting the experiments, the densities of mosquito larvæ,* Anophelines as well as Culicines, and of Cyclops† in the chambers were determined. After taking the densities on the forenoon of the 15th of December, 1940, a hundred specimens of *A. nama* were introduced into chamber *a*, and a hundred of *A. ranga* into chamber *b*. Chamber *c*, which was to serve as a control, was left untreated. Ladle dips of mosquito larvæ and tow-net collections of plankton were taken in the forenoon on every alternate day up to the 27th of December. Table III shows the records of observations made. As far as mosquito larvæ were concerned, the densities of Anophelines were lowered to some extent, i.e. from 3.9 to 2.0 in *a* and from 5.0 to 3.9 in *b*, while in *c* it varied between 4.2 and 9.2 with natural fluctuations (*vide* Job, 1941b). The Culicine densities were lowered from 6.9 to 1.9 in *a* and from 7.1 to 2.8 in *b*, while in *c* it fluctuated between 5.4 and 10.8. Considering the natural increase in larval population in the absence of fish in chamber *c*, the larvicidal activity of the glassfishes is not so small, as might appear if only the densities at the beginning and at the end of the experiment are compared. However, when compared with other larvicidal fishes of proved utility such as *Aplocheilichthys panchax*, their efficiency in this respect is very low.

As regards Cyclops, *A. nama* reduced their density from 11.6 to 0.0 in chamber *a* by the 25th of December. In chamber *b*, in which specimens of *A. ranga* were introduced, the reduction was more marked, for, though the initial density of 13.3 was higher than that in *a*, it was brought down to zero by the 21st of December. In chamber *c*, on the other hand, the density varied between 9.7 and 14.1.

* The density of mosquito larvæ was determined with larval dips as in the case of *Aplocheilichthys panchax* (*vide* Job, 1941b). The same ladle with a mouth area of 15.3 square inches and a volume of 22.5 cubic inches was used for taking dips of mosquito larvæ in all the experiments. On each occasion 20 dips were taken from each chamber, and after recording the larval counts, the contents were thrown back into the chamber.

† The density of Cyclops was determined by making plankton dips with a tow net. A small conical linen net, 12 inches wide and 20 inches deep, with a bamboo handle, 5½ feet long, was used. The net ended in a wide mouthed cylindrical bottle, 2.5 inches wide and 4 inches deep. After immersing the net to a depth of a foot and a half below the surface, it was gently taken out of the water while the plankton was collected in the bottle. Fifteen such dips were taken at different spots in each chamber. From the plankton thus collected in each dip three representative samples, each 1 cubic inch in volume, were pipetted out, and the rest thrown back into the chamber after all the dips were taken, so that the reduction of plankton population due to dipping was minimized. To the 3 cubic inches of plankton thus obtained, a few drops of formalin were added to preserve the microorganisms, and the Cyclops were later separated and counted in Petri dishes under a binocular microscope. By dividing the number obtained by 3, the number of Cyclops per cubic inch of the plankton collected in each dip was obtained. The average number per cubic inch of the 15 dips was taken as the density of Cyclops in the chamber.

of the Bombay Natural History Society added in an explanatory note, ' It seems clear that fingerlings of this fish feed largely on Cyclops but the question arises as to the extent to which Cyclops form the food of adult fishes The limitation of the destruction of Cyclops mainly to the fingerling stage in *Megalops* would reduce its utility as a destroyer of this pest, particularly as we have no evidence so far to indicate that *Megalops* breeds in fresh water '

Most small fishes and the larger species in their young stages may, when hungry, swallow edible little crustaceans like Cyclops either alone or along with other food Thus kullfishes, such as *Aplocheilichthys lineatus* (Pradhan, loc cit) and *A. panchax* (Sen, 1937, Job, 1941b), the striped Gourami, *Trichogaster fasciatus* (Sen, loc cit), and the spiny eel, *Mastacembelus pancalus* (Job, 1941a), have in some cases been found to feed on Cyclops, but these minute Copepods form only a very small percentage of their food

The requisites for a good cyclopocidal fish may be enunciated as follows —

- 1 The fish should be a feeder at all depths and a heavy consumer of Cyclops in nature throughout its life
- 2 It should not be of value as a food fish, but should be small in size and prolific in breeding so that both deep and shallow waters can be populated by large numbers of them
- 3 It should be active and agile so as to be able to escape its natural enemies, it should also be hardy and capable of transport and acclimatization in various waters
- 4 It should preferably be a widely distributed fresh-water species

As far as the first of these requirements is concerned, the observations made on the glassfishes show that they feed at all depths and that Cyclops* form a high percentage of their natural diet, sometimes almost to the exclusion of other elements, so that these fishes, with their specialized gill arches adapted for filtration, really stand out as voracious feeders of the pest throughout their life Being small and spiny, these glassfishes are eaten only by the poorest classes† The plentifulness of the fish is evident from the fact that during the season (October and November) basket loads are brought to the Ulubaria market and sold at only a couple of annas or sometimes even less per seer About one of the species, Innes (1935) writes, ' Shocking as it seems to those of us who treasure this little gem of the aquarium, it occurs in such large numbers in India and Burma that it is used as a fertilizer ' Considering the enormous numbers of eggs in their ovaries and

* Moorthy (1932) has found that on account of the comparatively sluggish movements of the infected Cyclops they are more easily fed on by fish than are non infected Cyclops He (Moorthy 1938) has also observed that infected Cyclops tend to go down to the bottom It is hence natural for the glassfishes which frequently roam in the lower layers, to consume enormous numbers of infected Cyclops

† Indiscriminate destruction of these small fishes, however, must be prevented in dracontial districts, especially in the epidemic season

nine specimens of each species were starved for twelve hours and then thrown into association with six spoonfuls of Cyclops in one of the experimental aquaria for half an hour. They were then captured and their gut-contents examined. Similarly, another set of nine specimens of each species were thrown into association with six spoonfuls of mosquito larvæ, mostly Culicines, and their gut-contents also studied. It was found that the maximum number of Cyclops consumed by *Ambassis nama* in half an hour was 327 per fish, and by *A. ranga* 468 per fish, while the corresponding numbers of mosquito larvæ were 24 and 19 respectively.

DISCUSSION

The significance of fishes in mosquito control has already been discussed by the author in his papers on the killifishes (*vide* Job, 1940b, 1941b). As far as the glassfishes are concerned, the results of the food analyses, field experiments and feeding experiments detailed above indicate that from the point of view of malaria control through the destruction of Anopheline mosquitoes, the glassfishes are inferior to the killifishes. The emphasis laid by some authors on the mosquitocidal values of the glassfishes was presumably based on the fact that under artificial conditions of an aquarium they consume comparatively large quantities of mosquito larvæ.

The great value of the glassfishes, however, lies in the control of guinea-worm disease* through their cyclopocidal activities. In this connection it may be remarked that modern methods of therapeutics and prophylaxis have been observed by various authors to be inadequate to cope with guinea-worm disease†. Evidently one of the best methods of successfully combating the guinea-worm on an extensive scale would be the biological method by the destruction of its intermediate hosts with the help of their natural enemies. The interesting investigations conducted by Pradhan (1930) and Moorthy and Sweet (1936) show that Cyclops are preyed upon by several fresh-water fishes, such as *Rasbora daniconius* (Ham.), *Nemachilus* sp., *Lepidocephalus thermalis* (Cuv. and Val.) and *Gambusia*. However, as Setna and Kulkarni (1940) note, the above-mentioned authors 'merely state the extent of the partiality of the various fishes for Cyclops, but they do not record what their investigations of the stomach contents of the fishes showed'. The importance of a study of the natural food and feeding habits of the species to be utilized for control work cannot be over emphasized. Again, some of the fishes listed by Pradhan and Moorthy, which were also dissected by Setna and Kulkarni (*loc cit*), are stated to have shown 'but insignificant quantities of Cyclops in their intestinal tract'.

Regarding the marine fish *Megalops cyprinoides* recently recommended by Setna and Kulkarni (*loc cit*) for guinea-worm control, the editors of the *Journal*

* As is well known, myriads of minute guinea worm larvæ discharged into water from the ulcer of an infected patient enter the digestive tract of certain species of Cyclops like *Mesocyclops leuckarti* and *M. hyalinus* (Moorthy, 1938), and reaching their hæmocoel, undergo some post larval changes there (Southwell and Kurshner, 1938), after which they are capable of further development in a human host. Man gets the infection by swallowing infected Cyclops with the drinking water.

† *Vide* Pradhan (1930), Moorthy (1932), Liston (1937) and Setna and Kulkarni (1940).

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the innumerable fry observed during the monsoon months, it may reasonably be assumed with Hora and Mukerji (1938) that the species 'breed very freely'. The numerous minute eggs, 'sprayed' on to submerged vegetation, hatch out in a day or two*, and the fry, growing quickly, attain maturity in a few months' time. These fishes are quick of movement and smart enough to evade their enemies. Further, being well protected by sharp spines in their median fins, larger fishes seldom swallow them. Although apparently delicate and small, the glassfishes, far from being tender, 'are fairly hardy' (Hora and Mukerji, *loc cit*), and the fact that they so successfully endure the necessary weeks of transport from Indian waters to American aquaria (*vide Innes, loc cit*) adequately proves their tenacity of life. Besides, being widely distributed, occurring in diverse types of water in different parts of the country, they also satisfy the condition of nearness mentioned by Prashad and Hora (*vide supra*) and can easily be dispersed in dracontial districts. Thus, the glassfishes satisfy most of the requirements of good cyclopscidal fish.

SUMMARY

Analyses of the gut-contents of two hundred and sixty-eight specimens of *Ambassis nama* and three hundred and thirteen of *A. ranga*, collected from their natural habitats in the malarious parts of Fuleshwar and Ulubaria in the Howrah district and Pulta in the 24-Parganas, showed that these glassfishes feed at all layers of water, subsisting mainly on minute Entomostracans which are found abundantly in the mid-water plankton. Their mouth and gill apparatus are well adapted for the filtration of such microfauna. To a small extent these fishes perform typical sight feeding also, and consume mosquito larvæ, mostly Culicines, from the sub-surface and worms from the bottom.

Field experiments conducted in railway borrowpits showed that the presence of the glassfishes checks mosquito breeding only to a small extent, while it effectively reduces the density of Cyclops.

Feeding experiments indicated that starved glassfishes under artificial conditions of the aquarium resort to sight feeding far in excess of feeding by filtration, and consume large, conspicuous organisms more than the microfauna.

The requisites for good cyclopscidal fish are listed. As most of these requirements are fulfilled by the glassfishes, and as no satisfactory species of fish appears to have so far been discovered for use against Cyclops, these fishes should prove of special value in the biological control of guinea-worm disease.

* The observations of Innes (*loc cit*), Mellen and Lanier (1935) and Stoye (1935) show that during the breeding time the glassfishes take a side to side position, quivering all the while and turning to an upside down pose, so that the eggs are discharged upward into fine leaved plants at the sub surface in lots of four to eight until forty or more have been laid. The small, clear and transparent eggs hatch out in 24 to 48 hours. The fry cling to plants or sides of other objects for two or three days, after which they resort to an active swimming life and grow quickly, feeding on fresh water micro-plankton.

TABLE II

Details of average percentages, (a) volumetric and (b) numerical, of food components in the gut-contents of two categories of Ambassis ranga (Hamilton)

Source of fish collection :—	1 Natural habitats	2 Experimental aquaria
Number of fish examined	313	12
Range of length	14 mm.—51 mm	18 mm —48 mm
Number of fish with empty gut	41	Nil
Portion of food digested beyond recognition	$\frac{1}{16}$	$\frac{1}{50}$

PERCENTAGE OF ITEMS IN RECOGNIZABLE ELEMENTS				
	1a Volumetric	1b Numerical	2a Volumetric	2b Numerical
Anophelines				
Early instar larvæ	3.43	0.30	2.67	1.50
Late instar larvæ	1.52	0.09	3.83	1.08
Pupæ	0.01	0.01		
Imagines				
Total anophelines	4.96	0.40	6.50	2.58
Culicines				
Early instar larvæ	2.83	0.89	18.50	5.33
Late instar larvæ	4.97	1.02	6.42	1.92
Pupæ	0.22	0.03		
Imagines				
Total Culicines	8.02	1.94	24.92	7.25
Total mosquito remains	12.98	2.34	31.42	9.83
Other insects	3.52	1.56	20.67	6.75
Total insect remains	16.50	3.90	52.09	16.58
Cyclops	42.89	71.13	21.33	69.25
Other Copepods	4.01	6.06		
Other crustaceans	25.73	17.27	6.33	14.00
Total crustacean remains	72.63	94.46	27.66	83.25
Miscellaneous animal remains	10.12	0.34	20.25	0.17
Total animal matter	99.25	98.70	100.00	100.00
Vegetable matter	0.37	1.24		
Inorganic matter	0.38	0.06		
Total	100.00	100.00	100.00	100.00

TABLE III

Effect of introducing glassfishes on the densities of mosquito larvae and Cyclops in borrowpit chambers

Date	AVERAGE ANOPHELINE DENSITY PER LADLE DIP			AVERAGE CULICINE DENSITY PER LADLE DIP			AVERAGE CYCLOPS DENSITY PER CUBIC INCH OF PLANETON COLLECTED		
	Chamber <i>a</i>	Chamber <i>b</i>	Chamber <i>c</i>	Chamber <i>a</i>	Chamber <i>b</i>	Chamber <i>c</i>	Chamber <i>a</i>	Chamber <i>b</i>	Chamber <i>c</i>
15-12-40	39	50	42	69	71	54	116	133	97
	<i>A nama</i> 100 introduced	<i>A ranga</i> 100 introduced	Left to serve as control	<i>A nama</i> 100 introduced	<i>A ranga</i> 100 introduced	Left to serve as control	<i>A nama</i> 100 introduced	<i>A ranga</i> 100 introduced	Left to serve as control
17-12-40	32	64	60	61	68	70	92	88	105
19-12-40	35	67	88	54	60	91	68	40	120
21-12-40	28	59	71	40	51	85	40	00	112
23-12-40	21	45	78	37	43	92	26	00	134
25-12-40	23	48	92	28	35	108	00	00	141
27-12-40	20	39	77	19	28	94	00	00	116

MYIASIS-PRODUCING DIPTERA IN MAN

BY

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AND

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[Received for publication, May 12, 1941]

FROM 1922 to 1941 a large number of collections of larvæ of the *Diptera muscoidea* from cases of myiasis were sent to us for report. They were mostly sent in a dead condition, but when they were living an attempt was made to breed them out to the adult stage on meat or fæces according to where they had been found.

Their identification and the localities from which they were collected are shown in Table I —

TABLE I

Reported habitat	Locality	IDENTIFICATION	
		Family	Species
Eye	Assam	Anthomyiidae	??
Nose	Bengal	Calliphoridae	<i>Chrysomya bezziana</i> , Vill.
			<i>Sarcophaga</i> sp
Ear	Calcutta	„	<i>Chrysomya bezziana</i> , Vill
Dental	Darjeeling	„	<i>Chrysomya bezziana</i> , Vill.
Mastoid region	,	,	<i>Sarcophaga duz</i> , Thoms

TABLE I—concl'd.

Reported habitat	Locality	IDENTIFICATION	
		Family	Species
Cutaneous	Bengal	Calliphoridae	<i>Sarcophaga ruficornis</i> , Fb <i>Sarcophaga dux</i> , Thoms <i>Chrysomya bezziana</i> , Vill
Inside dura mater of cord	Burma	"	* <i>Sarcophaga ceylonensis</i> , Park
Intestinal		"	<i>Sarcophaga ruficornis</i> , Fb
"	Bengal	"	<i>Sarcophaga dux</i> , Thoms
"	Assam	"	<i>Sarcophaga</i> sp
"	Bihar	"	† <i>Sarcophaga craggi</i> , Park
"	U P	"	<i>Drosophila</i> sp
"	Calcutta	Drosophilidae	<i>Apiochaeta scalaris</i> , Mg
"	"	Phoridae	

* Senior White (1924) is inclined to sink *S luzonensis* and *S ceylonensis* in *S dux*, Thoms

† Senior White (*loc cit*) thinks that *S craggi* is confined to Nyasaland and the Indian species *S dux*, Thoms, var *harpax* resembles *craggi* closely

The regions from which the specimens were collected are summarized in Table II —

TABLE II

Sites of infestation

Total number of samples received	Nasal	Ocular	Aural	Dental	Cutaneous	Intestinal	Other parts including penis	Not known
32	2	1	1	3	2	15	6	2

Of the different species enumerated above only one, *Chrysomya bezziana*, is biontophagous (Keilin, 1924) None of these patients, however, showed any particular symptom which could be ascribed to the presence of either biontophagous or saprophagous larvæ In a case of heavy infestation of the nose which was closely observed in the hospital, the patient merely complained of an uncomfortable sensation, there was no burning pain as was reported by Sinton (1921) In the

case of myiasis of a carious tooth reported by one of us (Strickland, 1929), the patient complained of toothache which was relieved after the maggots were collected and the cavity was cleaned. In another case of dental myiasis (Roy, 1934), the patient had inflammation of the gum which went on to abscess formation, the tooth was extracted and a large number of fly maggots emerged and were collected. He made an uneventful recovery.

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STUDIES OF GROUND WATER POLLUTION IN AN ALKALINE ALLUVIUM SOIL *

BY

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DURING the past ten years, many bored latrines have been installed in the East. Their popularity has been due to the ease of installation and cheapness of construction. However, questions have been raised, from time to time, as to the danger of contamination of shallow wells when the latrine extends down into the soil water. Much of the concern may have been due to the over-enthusiasm of some of the advocates of the bore-hole latrine, who have stated that the latrine might be put next to the family well.

Soils differ in texture and in alkalinity, and it was necessary for the public health officials in India to have data on the soil conditions of their own areas before approving the installation of bore-hole latrines in large numbers.

It was in the hope that conditions might permit of the safe introduction of the bore-hole latrine in rural areas, that an investigation (limited by considerations of funds available) was carried out by the Punjab Public Health Department with the object of determining, at least approximately, what the extent of the spread of pollution introduced into the sub-soil water might be.

The method of investigation was simple. A bore-hole was sunk to the level of the sub-soil water in an open space away from any known source of pollution. At measured distances from the bore-hole, hand pumps were introduced tapping the sub-soil water. Prior to the introduction of filth into the bore-hole, the quality of the sub-soil water drawn from the hand pumps was determined by repeated bacteriological and chemical examination. The quality of the water having been established, night-soil from a small community of 15 individuals was introduced into the bore-hole daily. Subsequent to this, regular examinations of the water from the pumps were carried out.

* The studies and observations on which this paper is based were conducted with the support and under the auspices of the International Health Division of the Rockefeller Foundation, in co-operation with the Department of Public Health, Punjab, India.

A material departure from the standard of purity of the water, established on examination prior to the introduction of the night-soil into the bore-hole, was regarded as due to the spread of pollution from the bore

From the observations made, pollution appeared to be capable of spreading from the point of introduction to a distance of 260 feet, in the direction of flow of the sub-soil water, in a period of four months

The limitations of the investigation were recognized, particularly because of the known wide variations in the quality of Punjab soil, but the results were so arresting that it was considered worth while that wider investigations should be carried out under more strictly controlled conditions

The objectives of the experiment here reported were —

- (a) To determine the extent and width of the pollution stream from an ordinary bored latrine extending below the water table, in certain areas of the Punjab plains
- (b) To determine whether the bore-hole latrine is practicable for the Punjab, and if so, what would be a safe distance between it and a shallow well situated at a point lower on the water gradient than the latrine
- (c) To derive a practical method for determining, within certain limits, the distance a well should be from a latrine, in these soils

Description of site A —The site decided upon for the experiment was in Model Town, which is about seven and a half miles from Lahore

The area is practically flat and almost bare of vegetation, and there is no well or latrine within a thousand feet. The soil crust extends to the water table, a depth of 15 feet, and below this are fine sand and silt, underlaid with quicksand

Owing to the constant clogging of the pumps at the first site selected it was impossible to get fundamental data, and it was therefore decided to move to a point where the quicksand lay at greater depth

Site D₁ —A 16-inch bore for a latrine was put down to 19 feet and cased to the water table. Five feet from the edge of the latrine, 9 pumps were placed in a circle 4 feet apart. The tops of the strainers averaged 17 feet 8 inches below the ground surface, or 2 feet 8 inches below the water table

Samples from the pumps and latrine at site D₁ were analysed for chloride alkalinity and pH. Silver nitrate determination was used throughout for chlorides

From the work at the first selected site extending over 2 months, it was found that there was no definite rise of salt concentration in pumps even when 20,000 grammes of salt were added to the latrine. After 500 c.c. of HCl were added however, the salt started to flow. Seven hundred and fifty cubic centimetres of HCl were then placed in the latrine, and the following day (6th December) 9,550 grammes of salt were added and agitated for 10 minutes with a compressor

The procedure followed throughout the experiment was to pump 4 16 Imperial gallons of water before taking samples

MODEL TOWN

EXPERIMENT D₁

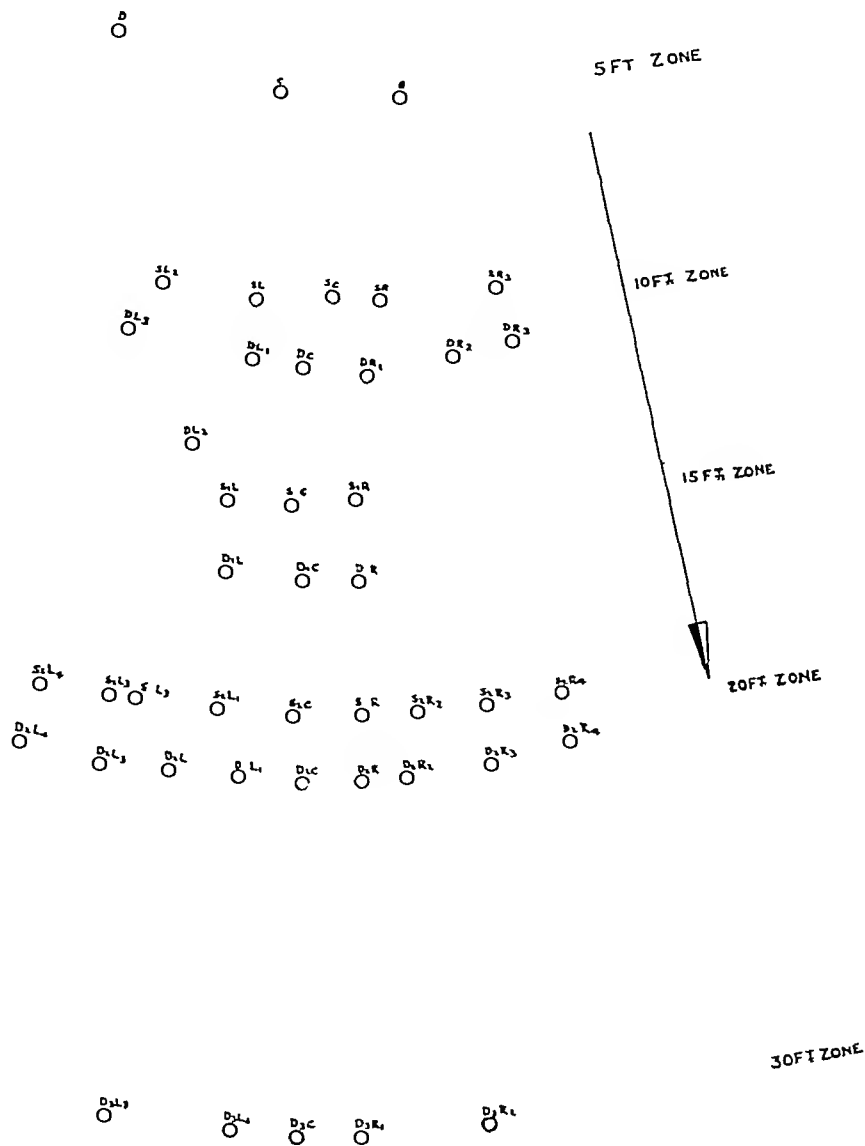


FIG 1 —Arrangement of wells at site D₁

Six days later, on 12th December, salt flowed strongly through pump C, having risen from 6.4 to 23.7 parts per 100,000. There had been a general slight rise, but besides C the principal rise had been in B, A and D. On the 14th, B, C and D were still rising, with C reaching 34.1, the other pumps were falling. The rise continued until the 19th, when the salt concentration dropped off suddenly in all pumps. Throughout this salt rise, the pH of all wells remained *unchanged*. It was unfortunate that our new conductivity meter did not work and we were thus unable to determine whether there was a rise of all salts.

The direction of flow thus established, the 10-foot zone was pegged out. Seven shallow wells were put down across the flow, 16 feet 5½ inches to the top of strainers. One and a half feet farther from the latrine seven deep wells, 18 feet 5½ inches to top of strainers, were put down.

Three shallow and three deep wells were put down in the 15-foot zone, nine shallow and nine deep wells in the 20-foot zone, and seven deep wells in the 30-foot zone. In case the stream was swinging to the right, extra holes were bored in the 30-foot and 40-foot zones so that pumps could be quickly installed.

The wells were numbered as follows —

5-foot zone	Wells, A, B, C, D, E
10-foot zone	SC, SR, SL, DC, DR, DL
15-foot zone	S ₁ C, S ₁ R, S ₁ L, D ₁ C, D ₁ R, D ₁ L
20-foot zone	S ₂ C, S ₂ R, S ₂ L, D ₂ C, D ₂ R, D ₂ L

Concrete platforms were laid around the pumps in all zones. All pumps were chlorinated until a residual chlorine reaction was obtained.

To check our findings at site A, it was decided to introduce salt without acid. On 6th January, 7,000 grammes of salt were put into the latrine and agitated and only a slight general rise resulted. On the 10th, 750 c.c. of HCl were added, and on the 13th 7,000 grammes of salt.

On the 25th there was a strong salt reaction in well B (22.1 parts per 100,000). On the 30th salt came through SR₃ of the 10-foot zone, which would seem to indicate that the stream was swinging to the right, with the velocity of flow at 0.83 feet per day.

Bacteriological tests — The procedure for the bacteriological examination of the water was as follows —

Five tubes containing 10 c.c. of double strength MacConkey medium are taken and each seeded with 10 c.c. of the original water.

One tube containing 5 c.c. single strength MacConkey medium is seeded with 5 c.c. of the original water.

PLATE XV



Type of concrete platforms laid around pumps

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PLATE XV



Type of concrete platforms laid around pumps.

Take two tubes containing 5 c c each of single strength MacConkey medium, in one put 1 c c of the original water, and in the second 0.5 c c of the original water

Add 1 c c of the original water for 9 c c of sterilized tap-water to make a dilution of 1 in 10

Now inoculate one tube containing 5 c c of single strength MacConkey medium with 1 c c of the above dilution

Incubate the tubes at 37°C and record the results after 24 and 48 hours

NB—The same procedure is followed for the Eijkman test except that the tubes are incubated at 45°C

If A+G is found in two of the five tubes inoculated with 10 c c of the original water it may be regarded as present in 50 c c of the water, if it is found in three or more tubes, it may be considered present in 10 c c of water

The sulphate reduction test is employed for detection of *Cl welchii*. The medium used is that recommended by Wilson and Blair. While still hot, 40 c c of the medium are added to an equal quantity of the water under examination and poured into a large Petri dish. This is incubated at 45°C, and the results are read after 24 and 48 hours

Results should be interpreted as follows —

- (a) Eijkman's test should be negative in quantities of water less than 50 c c
- (b) The presence of acid and gas in both MacConkey and Eijkman in equal small quantities of water should be regarded as an indication of recent pollution
- (c) The presence of acid and gas in small quantities of water in MacConkey but in larger quantities of water in Eijkman, would be regarded as an indication of remote pollution

In order to plot the results graphically, the following values were used —

Gas in two tubes of 10 c c	1 unit
Gas in three or more tubes of 10 c c	5 units
Gas in one tube of 5 c c	10 „
Gas in one tube of 10 c c	50 „
Gas in one tube of 0.5 c c	100 „
Gas in one tube of 0.1 c c	500 „

Water from the wells was examined every 3 or 4 days. The results of examinations given in Tables II to VII are representative

In the bacteriological examination of the wells in the 5-foot zone, well C was found to be polluted. It was re-chlorinated three or four times, and the third examination showed higher pollution than the first. However, the amount of

dissolved oxygen increased very considerably, and it was decided to seed the latrine with faeces. The 10-foot zone was free of contamination.

The latrine was seeded on 8th February. On the 13th all the wells of the 5-foot zone, including well C, were negative by both MacConkey and Eijkman tests. The 15-foot and 20-foot zones were tested and found free of contamination.

On 16th February contamination reached the 5-foot zone and wells A, B and C were found to be polluted, with the greatest pollution in well C. On the 23rd, only well C was positive to Eijkman and not so strongly as on the 16th. The 10-foot zone was negative.

On 1st March heavy floods washed under the concrete platforms in the 10-, 15- and 20-foot zones. These were repaired and chlorinated.

On 7th March wells B and C were positive to MacConkey in 10 c c and negative to Eijkman.

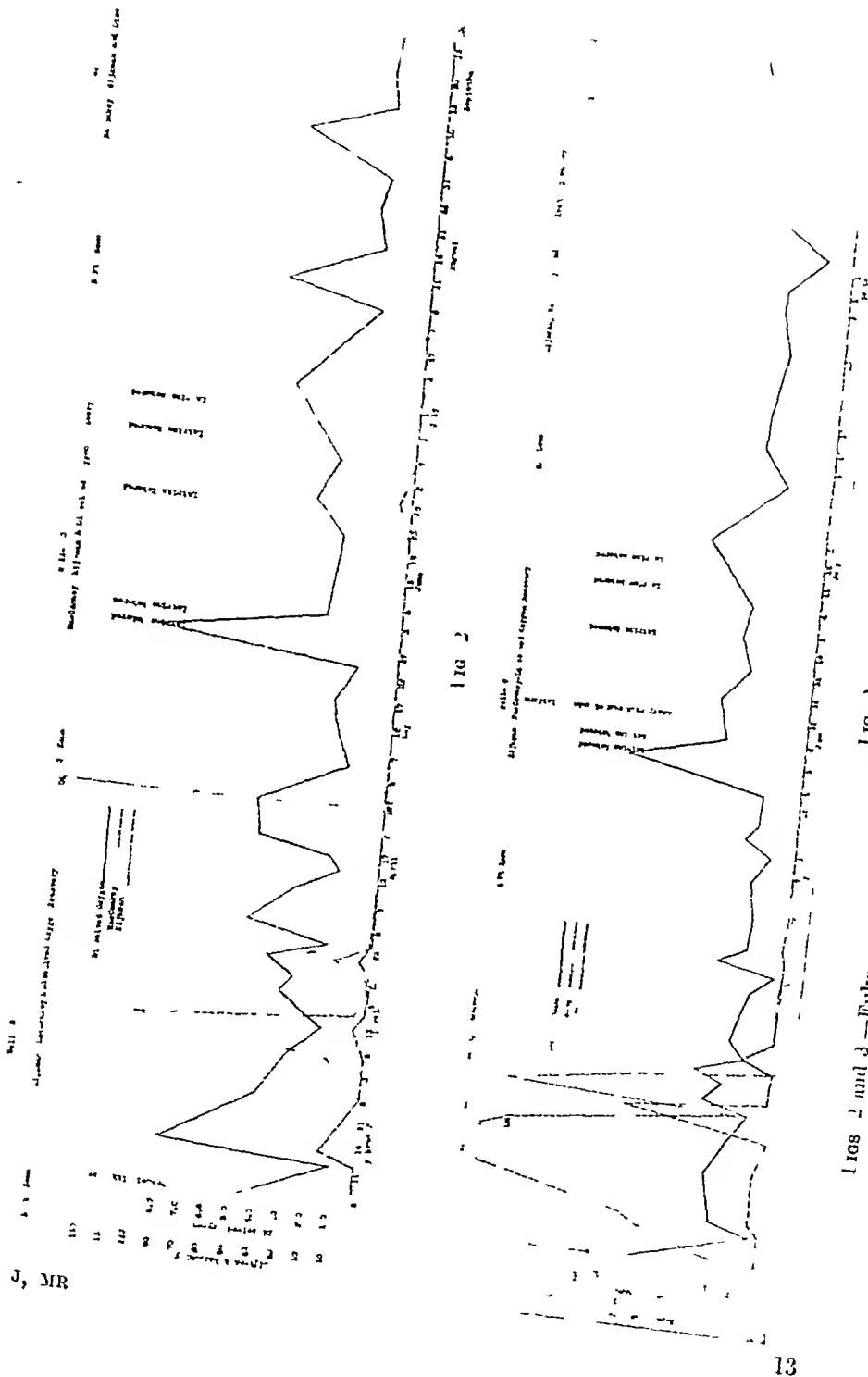
The water table had risen as the result of the flood and the level of the latrine was within 4 feet of the surface. On 13th March wells B and C were positive to Eijkman and MacConkey in 10 c c with C the more strongly polluted. The 10-foot zone remained negative. On 16th March wells B and C were positive to Eijkman in 50 c c indicating that the pollution stream had started to retreat again.

A new well was put down at a distance of $2\frac{1}{2}$ feet from the latrine but was negative on 20th March. Wells B and C were positive to MacConkey in 50 c c. On the 23rd all wells of the 5-foot zone were negative to both tests in 10 c c, while the $2\frac{1}{2}$ -foot zone was positive to Eijkman in 10 c c. On the 27th wells B and C were positive to Eijkman in 10 c c. On 30th March all wells in the 10-foot zone were negative to Eijkman in 10 c c.

On 6th, 7th and 10th April, tests were made of the 10-, 15- and 20-foot zones, and all were found to be negative to Eijkman. On 14th April all wells of the $2\frac{1}{2}$ - and 5-foot zones were negative.

The $2\frac{1}{2}$ -, 5- and 10-foot zones remained negative to Eijkman up to and including 26th May, but on the same day the considerable jump in the MacConkey tests showed increased contamination in the $2\frac{1}{2}$ -foot zone. For that reason it was thought probable that the charging latrine had become partially filled with silt, as the level had reached within 4 feet of the surface. A sample showed that silt and faeces were mixed together. The latrine was re-bored to $18\frac{1}{2}$ feet on 29th May. Careful watch was kept on the charging latrine, and as it appeared to be filling again it was re-bored on 5th June, when fresh faeces were noticed in the latrine. A policy was then adopted of keeping the charging latrine at all times between 18 and 19 feet deep.

Tests on 22nd June showed all zones negative to Eijkman. Tests on 28th June and 6th July showed negative results in all zones. During the next three weeks heavy rains occurred, and the whole area was flooded several times, including



the portion under the platforms in the 5-, 10- and 15-foot zones. Repairs were made and the wells disinfected.

The 2½-foot zone became positive to Eijkman in 50 c c on 31st July, but became negative on 17th August. It became positive again on 25th August in 5 c c and dropped from 30th August to be again contaminated in 50 c c in September. It rose again to positive in 10 c c on 19th of September and continued so until the end of the experiment on 29th of September. During this period, the 5- and 10-foot zones were negative. MacConkey tests were also negative in the 5- and 10-foot zones during the period.

Cl. welchii was not encountered at any time during the experiment. The great variation between MacConkey and Eijkman tests, as shown in Figs 2, 3 and 4, demonstrate either that MacConkey reacted to many other organisms besides faecal ones or that Eijkman was not sufficiently delicate a test for determination of faecal contamination of water. It will be noted from the graphs that with a few exceptions the dissolved oxygen confirmed slightly more with the MacConkey test than with the Eijkman.

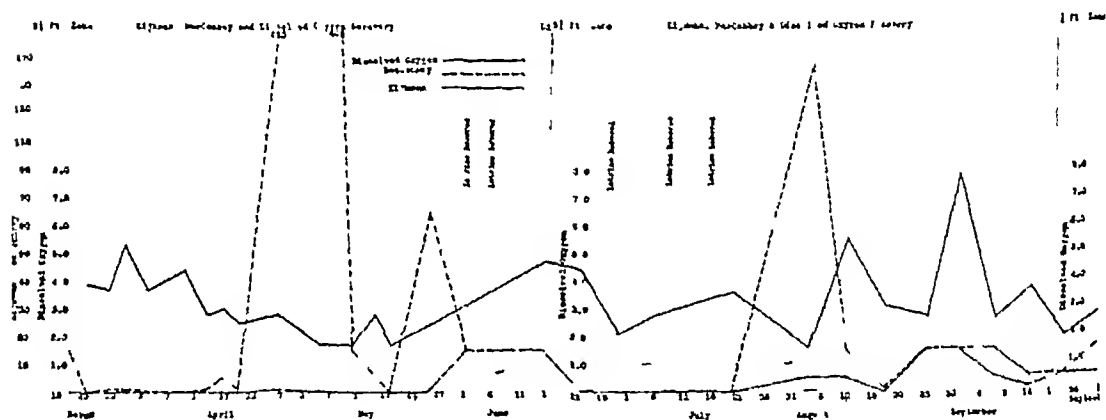


FIG. 4.—Eijkman and MacConkey tests and dissolved oxygen recovery in 2½ foot zone

It is quite clear from the accompanying data that faecal pollution travelled slowly in the direction of flow to an extent of over 5 feet, but not as far as 10 feet. As the pore spaces in the soil became clogged, faecal pollution retreated towards the latrine. It extended again when severe rains raised the water surface in the wells so that the water could travel through fresh soil and retreated when the pores again became clogged. The extent of bacteriological pollution as a result of re-boring at no time extended as far as the first soil contamination. In other words, it passed the 2½-foot zone but never reached the 5-foot one.

Physical and chemical tests—It should be remembered that in this type of highly alkaline soil the reactions were quite different from those which had been previously encountered in acid or neutral soils. At no time during the experiment was there any variation in the pH as related to pollution. There was no odour or

foaming. Absence of chlorides seems to have little bearing upon the degree of pollution. Nitrates were usually absent when pollution was found in different wells, but nitrites were present more often than not. In other words, the nitrogen cycle appeared to be interrupted. Free and albuminoid ammonia tests were not run and so no comparison could be made on this point. From the chemical standpoint the only dependable factor to check the bacteriological findings was dissolved oxygen, which proved satisfactory.

Chemical recovery—Nitrates only were found in the 5-foot zone before the addition of faeces and after chlorinating the latrine, no nitrates or nitrites were

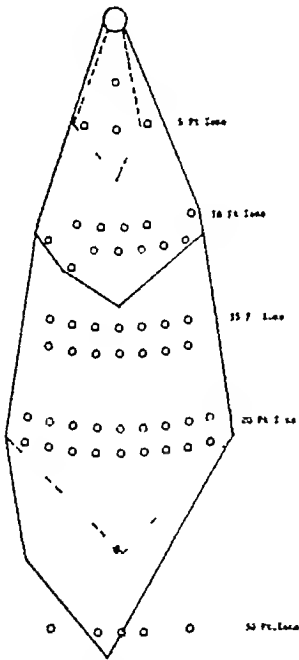


FIG 5 A—Extent and retreat of chemical contamination, D₁ area.

Shallow wells -----
Deep wells -----
2½ ft. zone.

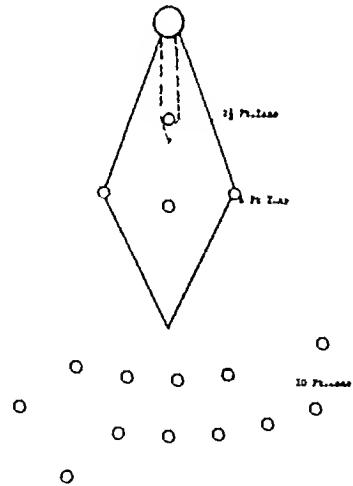


FIG 5 B—Extent and retreat of bacteriological contamination, D₁ area.

found until heavy floods occurred and washed under the platforms. All wells were chlorinated after repairs were made and, as shown in Table IV, nitrites and nitrates were present during the period the pollution was retreating.

Attention is drawn to (a) the relationship between the dissolved oxygen and the nitrites, (b) the little change in pH, (c) the fact that in the beginning reaction to phenolphthalein had to be expressed as acidity, changed gradually to neutral and then expressed as alkalinity (*methyl* orange was unsatisfactory, as

the end-point was too indefinite), and (d) the disappearance of nitrates towards the end of the experiment

Water gradient—It had been previously ascertained by the Irrigation Department that the gradient of the soil water was approximately 1 in 5,000. To determine the water gradient for ourselves, we installed four observation wells. One was placed 5 feet from the charging well, another 100 feet higher up on the water gradient, a third 100 feet below the charging well on the water gradient, and the last 300 feet below the charging well on the lower part of the gradient. From the plotted curves it would seem that in pre-monsoon conditions the water gradient was 1 in 1,600, in mid-monsoon it was 1 in 530, and post-monsoon 1 in 640.

However, it will be noticed from a study of the curves that there was great variation in the observation wells and it would seem at times that the wells were not placed parallel to the direction of flow of soil water. (However, from the daily readings, a conclusion was reached similar to that of the Director of the Irrigation Research Institute, Punjab, in his Annual Report for the year ending April 1939.)

Our experiment was conducted wholly in what is termed the 'crust'. The clay content at 15 feet was 18.5 per cent, at 18 feet 2 inches it was 23.3 per cent, and at 21 feet 7 inches it was 12.5 per cent. (The pH varied from 9.18 to 9.42, which appears to be within the limits set by the Director of the Irrigation Research Institute.)

Frequent borings were made around the charging well where the thickness of the 'crust' varied from 21 feet to 30 feet in the line of the direction of flow. It appeared to be undulating, and the observation well within 5 feet of the charging well appeared to have a deeper crust. Greater upward pressure was exerted at this place than in other wells, and it was decided that the readings of this observation well should be discounted because of its variations in water-table elevations from day to day.

It is therefore impossible under these circumstances to state definitely the true gradient of the soil water, as the data would be non-conclusive unless the borings were carried through the crust in every case and the depth of the crust noted.

SUMMARY

The findings here reported relate to special soil conditions prevailing throughout most of the Punjab. They apply only to the upper soil, or *crust*, which has a high alkalinity and a high percentage of sodium sulphate. The soil is typical of irrigated areas, where indiscreet irrigation methods have been followed and the land has become useless for field crops.

The conclusions must not be confused with those based on investigations conducted in quicksand lying below the crust.

The previous experiment by the Department of Public Health was conducted in quicksand, therefore its results are not comparable with those of the study reported here.

It has been pointed out by the Director of the Irrigation Research Institute that there is a great deal of kankar (lumps of an impure form of calcium carbonate) in the Punjab soils, and that the addition of hydrochloric acid would dissolve the kankar, thus opening up passage in the soil through which flow would take place

This possibility was recognized as was the fact that the extent of pollution would be greater than under natural conditions. However, after the passages were opened with acid, the contamination did not extend 10 feet from the latrine

The established routine was faithfully followed —

- (a) A petrol tin of water (4 16 Imperial gallons) was pumped from each well before sampling
- (b) Two gallons of fresh faeces was put into the latrine daily
- (c) Fixation for dissolved oxygen was carried on at the site
- (d) pH buffer tubes were checked regularly

The extent of pollution, as determined by the Eijkman method, passed the 5-foot zone but did not reach the 10-foot zone

The extent of pollution, as shown by the MacConkey method, passed the 15-foot zone but did not reach the 20-foot zone

After retreat and subsequent advance pollution, as shown by the Eijkman and MacConkey tests, passed the 2½-foot zone but did not reach the 5-foot zone

ACKNOWLEDGMENTS

Grateful credit must be given to the collaborators in the experiment reported above, which entailed great patience and hard work in the scorching sun to Dr S M Rafi, M B, B S, D P H, who assisted from October 1938 to 27th March, 1939, to Dr D R Mehta, M B, B S D P H, Assistant Epidemiologist, Punjab, who took over from 27th March to the end of the experiment and carried efficiently a heavy part of the work, to Dr A Sarup, M Sc, Ph D, for his efficient chemical work, to Mr B M Kapur, Chief Sanitary Inspector, Punjab, for most intelligent handling of field staff and for his work as technician, to Dr M Yacob, M B, B S, D P H, D Bact, Assistant Director of Public Health, who set the standards and criticized and checked all bacteriological findings, to Dr E McKenzie Taylor, Director of Irrigation Research Institute, Punjab, for his valuable guidance, to Mr D A Howell, Superintending Engineer and Sanitary Engineer, Punjab, for advice as to conditions, and to Lieut-Colonel C M Nicol, F R S, Director of Public Health, Punjab, for his whole-hearted co-operation in selecting men and for the use of his laboratories and staff. This investigation would never have been made if the Director had not been convinced from his own investigations that there was a great danger of deep latrines being more of a menace than a boon to rural communities

TABLE I

Direction of ground water flow from latrine, NaCl as indicator (5-foot zone)

Well	Chloride	pH	Chloride	pH	Chloride.	pH
	December 6		December 12		December 14	
LT	795.3	3.8	11.748	7.2	10.640	7.4
A	6.4	7.6	10.5	7.6	11.3	7.6
B	6.9		10.5	7.6	11.7	7.6
C	6.4	7.6	23.7	7.6	34.1	7.6
D	6.4	7.6	12.7	7.7	9.7	7.6
E	6.3	7.6	11.0	7.6	9.9	7.6
F	6.3	7.7	10.2	7.7	11.0	7.7
G	6.4	7.8	13.1	7.7	10.0	7.7
H	6.3	7.7	9.9	7.6	9.0	7.6
I	6.3	7.6	10.7	7.6	9.0	7.5
	December 16		December 17		December 19	
LT	10.138	7.6				
A	9.9	7.6	9.4		10.5	
B	26.3	7.8	17.7		10.0	
C	12.4	7.6	37.4		9.9	
D	8.5	7.7	8.7		11.2	
E	8.2	7.6	10.7		12.0	
F	9.0	7.8	10.8		13.0	
G	8.4	7.7	10.0		9.0	
H	9.0	7.6	8.5		8.2	
I	10.0	7.6	8.5		9.2	

780 cc of HCl added to LT on December 5

9,550 g of NaCl added to LT on December 6

TABLE II

Analyses of samples from wells before latrine was seeded with faeces (5-foot zone)

Well	pH	Alkaline or acid	Cl ₂	NO ₂	NO ₃	Dissolved oxygen	50 cc	TIME RECOVERY							
								10.00	5.00	10.00	5.00	10.00	5.00	10.00	5.00
								E	M	I	M	I	M	I	M
							E*								
February 6															
A	7.0	20.0	11.0	0.0	0.0	1.8	-	-	-	-	-	-	-	-	-
B	7.7	30.0	10.7	0.0	0.124	4.0	-	-	-	-	-	-	-	-	-
C	7.7	52.0	9.2	0.0	0.118	5.7	+	+	+	-	-	-	-	-	-
D	7.7	30.0	9.0	0.0	0.0	2.2	-	-	-	-	-	-	-	-	-
E	7.0	30.0	8.0	0.0	0.120	3.2	+	-	+	-	-	-	-	-	-
H	7.0	30.0	15.2	0.0	0.0	3.2	-	-	-	-	-	-	-	-	-
I	7.0	30.0	10.0	0.0	0.0	2.0	-	-	-	-	-	-	-	-	-
J	+9.0	144.0	15.0	0.0	0.94	5.4	-	-	-	-	-	-	-	-	-
IT	7.1	352.0	2.203	0.0	0.00	2.4	-	-	-	-	-	-	-	-	-

* E = Eijkman

† M = MacConkey

Wells C and E were negative on February 13

TABLE III

Analyses of samples from wells before latrine was sealed with faeces (10-foot zone)

Well	pH	Alkaline or acid	Cl ₂	NO ₂	NO ₃	Dissolved oxygen	TUB RECOVERY									
							50 cc		10 cc		5 cc		10 cc		5 cc	
							F*	M†	F	M	F	M	F	M	F	M
SC	7.6	20.0	11.5	Trace			-	-	-	-	-	-	-	-	-	-
SR ₁	7.6	24.0	9.7	0.0	0.0	4.7	-	-	-	-	-	-	-	-	-	-
SR ₂	7.6	26.0	11.3	0.0	0.0	3.4	-	-	-	-	-	-	-	-	-	-
SL ₂	7.6	24.0	8.7	0.0	0.0	3.2	-	-	-	-	-	-	-	-	-	-
DC	7.6	20.0	8.9	0.0	0.0	1.7	-	-	-	-	-	-	-	-	-	-
DR ₁	7.6	26.0	9.4	0.0	0.0	3.2	-	-	-	-	-	-	-	-	-	-
DR ₂	7.6	20.0	8.7	0.0	0.1	1.9	-	-	-	-	-	-	-	-	-	-
DR ₃	7.6	34.0	8.4	0.0	0.0	1.3	-	-	-	-	-	-	-	-	-	-
DL ₁	7.5	22.0	9.0	0.0	0.0	2.6	-	-	-	-	-	-	-	-	-	-
DL ₂	7.5	54.0	8.2	0.0	0.0	3.8	-	-	-	-	-	-	-	-	-	-
DL ₃	7.5	26.0	8.4	0.0	0.0	3.7	-	-	-	-	-	-	-	-	-	-

* F = Frikman

† M = MacConkey

TABLE IV
Analyses of samples from wells after latrine was sealed with faeces (5-foot zone)

Well	pH	Alkaline or acid	Cl ₂	NO ₂	NO ₁	Dissolved oxygen	TUBS MACONKEY									
							50 cc		10 cc		5 cc		10 cc		5 cc	
							P†	M†	F	M	E	M	E	M	E	M

February 16																
A	7.6	32.2*	10.2	-	-	0.3	+	+	+	+	+	+	+	+	+	+
B	7.7	20.0*	0.2	-	-	8.0	+	+	+	+	+	+	+	+	+	+
C	7.5	20.0*	8.5	-	-	2.3	+	-	+	+	+	+	+	+	+	+
D	7.6	30.0*	8.6	-	-	3.7	-	-	-	-	-	-	-	-	-	-
I	7.6	42.0*	8.2	-	-	3.7	-	-	-	-	-	-	-	-	-	-
II	7.5	30.0*	8.0	-	-	4.4	-	-	-	-	-	-	-	-	-	-
I	7.6	38.0*	9.4	-	-	8.2	-	-	-	-	-	-	-	-	-	-
Ob	+0.6	24.4	23.4	-	-	3.9	-	-	-	-	-	-	-	-	-	-

February 27																
B	7.7	38.0	8.4	-	-	4.2	-	-	-	-	-	-	-	-	-	-
C	7.6	28.0	8.0	-	-	2.7	+	+	+	+	+	+	+	+	+	+
D	7.7	32.0	8.2	-	-	1.6	-	+	+	+	+	+	+	+	+	+

* Acidity in CaCO₃ in parts per million

† E = Fykman

† M = MacConkey

TABLE IV—*concl'd.*

Well	pH	Alkaline or acid	Cl ₂	NO ₂	NO ₃	Dissolved oxygen	TUBE RECOVERY											
							50 cc		10 cc		5 cc		10 cc		05 cc		01 cc	
							E†	M†	E	M	E	M	E	M	E	M	E	M
March 13																		
B	81	490*	123	No	No	19	+	+	+	+	+	+	+	+	+	+	+	+
C	75	300*	169	No	No	10	+	+	+	+	+	+	+	+	+	+	+	+
D	75	280*	99	No	No	13	-	-	-	-	-	-	-	-	-	-	-	-
March 23																		
2½-foot zone	81	440	155	0011	00	39	-	-	+	-	-	-	-	-	-	-	-	-
B	83	360	105	00723	Trace	31	-	+	-	-	-	-	-	-	-	-	-	-
C	76	100*	141	00841	0071	36	-	+	-	-	-	-	-	-	-	-	-	-
D	76	220*	92	00158	00	19	-	-	-	-	-	-	-	-	-	-	-	-
* Acidity in CaCO ₃ in parts per million																		
† E = Eijkman † M = MacConkey																		

* Acidity in CaCO₃ in parts per million

† E = Ejikman

‡ M = MacConkey

TABLE V
Analyses of samples from wells after latrine was seeded with faeces (10-foot zone)

Well	pH	Alkaline or acid	Cl ₂	NO ₂	NO ₃	Dissolved oxygen	TUBE RECOVERY											
							50 00		10 00		50 000		1 00		05 00		01 00	
							E†	M†	E	M	E	M	E	M	E	M	E	M
March 8																		
SC	7.0	8.0*	9.7	0.0033	0.3544	3.0	-	-	-	-	-	-	-	-	-	-	-	-
SR ₁	8.3	34.0	28.7	0.0633	0.1417	3.3	-	-	-	-	-	-	-	-	-	-	-	-
SR ₃	7.7	32.0	18.6	0.058	0.1772	2.0	-	-	-	-	-	-	-	-	-	-	-	-
SL ₄	7.7	14.0*	18.3	0.0387	0.212	1.4	-	+	-	+	-	+	-	-	-	-	-	-
DC	7.7	38.0*	16.1	0.0188	0.0	1.7	-	+	-	+	-	-	-	-	-	-	-	-
DR ₁	7.7	24.0*	13.2	0.0435	0.248	2.3	-	+	-	-	-	-	-	-	-	-	-	-
DR ₂	8.1	52.0	11.5	0.041	0.1417	2.8	-	+	-	-	-	-	-	-	-	-	-	-
DR ₃	+9.6	80.0	12.5	0.0096	0.2126	2.8	-	-	-	-	-	-	-	-	-	-	-	-
DL ₁	7.6	20.0*	11.2	0.0316	0.092	2.8	-	+	-	-	-	-	-	-	-	-	-	-
DL ₂	7.6	28.0*	12.2	0.0279	0.106	1.2	-	-	-	-	-	-	-	-	-	-	-	-
DL ₃	7.6	34.0*	46.8	Trace	0.0	2.3	-	+	-	+	-	+	-	-	-	-	-	-

† M = MacConkey

† E = Eijkman

* Acidity in CaCO₃ in parts per million

TABLE V—*concd*

Well	pH	Alkaline or acid	Cl ₂	NO ₂	NO ₃	Dissolved oxygen	TUBE RECOVERY											
							50 cc		10 cc		50 cc		1 cc		0.5 cc		0.1 cc	
							E†	M†	E	M	E	M	E	M	E	M	E	M
March 23																		
SC	7.9	20.0	9.9	0.0512	0.1417	3.0	-	-	-	-	-	-	-	-	-	-	-	-
SR ₁	8.1	44.0	11.3	0.0447	0.0921	3.5	-	-	-	-	-	-	-	-	-	-	-	-
SR ₂	7.7	8.0*	20.6	0.0394	0.1063	1.1	-	-	-	-	-	-	-	-	-	-	-	-
SL ₂	7.8	18.0	14.3	0.05	0.3543	4.3	-	-	-	-	-	-	-	-	-	-	-	-
DC	7.6	16.0*	16.0	0.0434	0.0567	2.7	-	-	-	-	-	-	-	-	-	-	-	-
DR ₁	7.7	14.0*	13.3	0.0421	0.0	1.4	-	-	-	-	-	-	-	-	-	-	-	-
DR ₂	7.6	16.0*	11.2	0.0447	0.0	2.3	-	-	-	-	-	-	-	-	-	-	-	-
DR ₃	8.1	38.0	9.0	0.054	0.0	3.9	-	-	-	-	-	-	-	-	-	-	-	-
DL ₁	7.7	14.0*	9.2	0.05	0.05	7.9	-	-	-	-	-	-	-	-	-	-	-	-
DL ₂	7.6	20.0*	11.2	0.0473	0.0354	1.0	-	-	-	-	-	-	-	-	-	-	-	-
DL ₃	7.6	30.0*	20.7	0.0158	0.0	3.2	-	-	-	-	-	-	-	-	-	-	-	-

* Acidity in CaCO₃ in parts per million

† E = Eijkman.

† M = MacConkey

TABLE VI

Analyses of samples from wells (5-foot and 10-foot zones)
(July 20 to September 28)

Well	Dissolved oxygen	pH	Alkaline or acid	Cl ₂	NO ₂	NO ₃
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July 20

2½ foot zone	3.65	+8.4	40.0	46.2	+	—
5 foot zone						
B	5.05	+8.4	76.0	65.3	—	—
C	4.5	7.7	9.0	10.2	Trace	+
D	4.7	7.7	5.0	9.2	+	—

July 20

10 foot zone						
SC	4.3	8.3	20.0	9.9	+	Trace
SR ₁	3.9	8.3	22.0	11.8	Trace	,
SR ₂	5.0	8.4	20.0	9.2	—	—
SL ₂	—	—	—	—	—	—
DC	2.5	7.9	6.0	7.2	Trace	Trace
DR ₁	2.1	8.1	3.0	7.9	„	—
DR ₂	3.8	8.1	7.0	7.5	+	—
DR ₃	3.5	7.7	4.0	7.2	Trace	—
DL ₁	3.5	7.7	8.0	7.2	+	Trace
DL ₂	3.7	7.7	4.0	7.2	Trace	—
DL ₃	3.9	7.7	9.0	12.2	+	—

TABLE VI—contd

Well.	Dissolved oxygen	pH	Alkaline or acid	Cl ₂	NO ₂	NO ₃
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August 17

2½-foot zone	3 1	+8 4	90 0	41 2	Trace	—
5 foot zone						
B	2 0	+8 4	176 0	16 4	Trace	—
C	3 0	7 9	4 0	9 8	„	—
D	2 7	7 7	Neutral	10 8	„	—

August 17

10 foot zone						
SC	3 9	8 1	Neutral	11 9	Trace	—
SR ₁	2 9	8 4	22 0	7 9	„	—
SR ₃	5 7	+8 4	26 0	6 6	„	—
SL ₁	3 2	+8 4	210 0	24 2	„	—
DC	3 7	8 1	2 0	7 9	—	—
DR ₁	2 6	8 3	14 0	7 9	—	—
DR ₂	3 6	8 1	12 0	8 6	—	—
DR ₃	2 8	7 9	Neutral	7 6	—	—
DL ₁	2 4	7 9	„	7 2	—	—
DL ₂	2 7	7 9	„	8 2	—	—
DL ₃	4 2	7 7	„	15 6	—	—

TABLE VI—*concl'd*

Well	Dissolved oxygen	pH	Alkaline or acid	Cl ₂	NO ₂	NO ₃
September 28						
2½ foot zone	27	+84	640	445	Trace	—
5-foot zone						
B	20	+84	2260	136	—	—
C	29	77	Neutral	106	0 0118	—
D	30	75	160*	119	0 0065	—

September 28

10 foot zone						
SC	27	79	Neutral	95	0 0775	0 2125
SR ₁	34	82	300	73	—	—
SR ₂	40	82	240	66	—	—
SL ₂	—	+84	1800	165	—	—
DC	49	75	Neutral	73	0 0919	—
DP ₁	20	81	280	69	—	—
DR ₂	31	77	Neutral	73	0 0315	—
DR ₂	36	77	"	66	Trace	—
DL ₁	27	75	80*	73	0 0328	—
DL ₂	30	77	Neutral	79	0 0210	—
DL ₂	50	75	160*	119	0 0591	0 1063

* Acidity in CaCO₃ in parts per million

J, MR

August 2

2½ foot zone	+8.4	70.0	41.4	Trace	-	1.0	+	+	+	+	-	+	-	+	-	-
5 foot zone																
B	+8.4	172.0	18.0	-		1.0										
C	7.7	8.0	9.5	Trace	-	2.0										
D	7.0	6.0	9.9	0.0158	-	2.1										

September 28

2½ foot zone	+8.4	64.0	44.5	Trace	-	2.7	+	+	+	+	+	+	-	-	-	-
5 foot zone																
B	+8.4	220.0	13.0	-		2.0										
C	7.7	Neutral	10.0	0.0118	-	2.0										
D	7.5	10.0†	11.9	0.0065	-	3.0										

* E = Eukman

† M = MacConkov

† Acidity in CaCO_3 in parts per million

SODIUM CHLORIDE AS AN INDICATOR FOR THE DETERMINATION OF THE FLOW OF SOIL WATER *

BY

BRIAN R DYER

[Received for publication, May 23 1941]

IN a previous paper (Dyer, 1941) it was mentioned that at two experimental sites, A and D₁, when salt alone was added to the charging well to determine the direction of underground water flow, no recovery of salt was secured in the surrounding wells even after the lapse of several weeks. However, when 1 or 2 pounds of hydrochloric acid were introduced into the charging well and salt was added the following day, the salt was recovered in surrounding wells so that the direction of flow and subsequent velocity could be determined.

The same experience was encountered at A₁, 250 feet from D₁. The soils at both of these sites were highly alkalized, the lowest pH in any stratum being 8.4, but D₁ was in alluvium and A₁ was in quicksand. It was decided to check this phenomenon very carefully by a new experiment at Karol, at a location about 15 miles from the sites of the other experiments. The soil was less alkaline and somewhat coarser in character but the screens of the pumps were well down into the quicksand. Results of mechanical soil analyses made at field A₁ and at Karol are shown in Tables I and II —

TABLE I
Mechanical soil analyses at A₁

Distance from surface —	15'	15'-16' 6"	16' 6"-18' 8"	18' 8"-20' 10"
Effective size	0.043	0.071	0.09	0.051
Uniformity coefficient	5.1	2.7	2.2	3.6

* The studies and observations on which this paper is based were conducted with the support and under the auspices of the International Health Division of The Rockefeller Foundation, in co operation with the Department of Public Health, Punjab, India

TABLE II

Mechanical soil analyses at Karol (opposite well MD)

Distance from surface in feet —	0-1	3-4	5-6	8-9	12-13	14-16	16-18	18-20	20-22
Effective size	0.061	0.04	0.075	0.045	0.1	0.087	0.19	0.125	0.175
Uniformity coefficient	1.9	4.6	2.2	3.6	3.1	2.0	2.2	2.4	2.1

Kankar (lumps of impure calcium carbonate) is widespread in this part of the Punjab, but at site A₁ it was present only in the upper 2 feet of soil and at Karol it was found only 18 feet below ground surface. This fact is rather significant in that the first salt reaction at site A₁ was secured in the shallow wells of the 5-foot zone, while at Karol salt reaction occurred only in the deep wells of the 10-foot zone. There was no salt reaction at any time in the shallow or medium deep wells.

The results of chemical analyses of soil samples at A₁ after the addition of salt are given in Table III. At Karol specimens were taken from four sides of the charging well before the addition of salt. Table IV gives the analyses of soil samples below the charging well in the direction of flow.

TABLE III

Analyses of soil samples (after adding salt) at A₁, in 10-foot zone, in direction of flow

Depth in feet	pH Soil water 1 5	Total salts (per cent)	Clay content (per cent)	EX BASES, MGS PER 100 g			Moisture
				Ca	Mg	Na and K	
0-1	10.2	1.45	21.12	4.40	0.5	14.5	13.35
1-2	10.1	1.60	19.20	8.20	0.4	12.1	15.41
2-3	10.15	1.50	17.28	3.80	0.6	10.2	18.10
3-4	10.2	1.38	15.26	4.60	0.6	11.0	20.28
4-5	10.3	1.30	20.28	3.0	0.5	14.7	22.5
5-6	10.25	0.63	26.32	2.80	0.4	15.0	24.20
6-7	10.2	0.30	14.64	7.80	0.3	8.3	20.46
7-8	10.15	0.22	15.78	8.80	0.2	6.6	22.87
8-9	10.15	0.183	11.70	8.00	0.2	8.6	18.14

TABLE III—concl'd

Depth in feet	pH Soil water 1 5	Total salts (per cent)	Clay content (per cent)	EX BASES, MCS PER 100 g			Moisture
				Ca	Mg	Na and K	
9-10	10.10	0.21	9.74	3.2	0.2	5.8	14.60
10-11	9.80	0.107	2.20	4.0	0.0	2.8	4.43
11-12	9.70	0.103	3.08	4.6	0.0	2.4	5.88
12-13	9.70	0.107	3.12	5.0	0.0	2.8	10.66
13-14	9.80	0.095	2.64	4.0	0.0	2.2	17.75
14-15	9.70	0.076	3.64	3.4	0.0	2.0	27.24

Water table

15-16	9.85	0.123	2.20	6.4	0.0	2.8	
16-17	9.70	0.088	4.58	4.6	0.0	2.4	

TABLE IV

*Analyses of soil samples at Karol between the 5-foot and 10-foot zones,
7 feet 6 inches from the charging well (opposite well MH)*

Depth in feet	pH Soil water 1 5	Total salts (per cent)	Clay content (per cent)	EX BASES, MCS PER 100 g			Moisture
				Ca	Mg	Na and K	
0-1	8.45	0.145	54.46	11.60	0.2	0.6	10.8
1-2	8.45	0.120	31.46	12.20	0.2	0.8	10.5
2-3	8.75	0.082	12.88	10.60	0.3	1.70	11.3
3-4	8.70	0.064	11.40	7.40	0.4	1.80	18.4
4-5	8.80	0.074	8.16	2.40	0.4	2.20	22.2
5-6	8.25	0.064	6.28	11.0	0.2	1.90	26.3
6-7	8.50	0.066	6.80	11.8	0.3	1.30	26.5

pieces of the original basket which had been saved for the purpose were soaked for a fortnight in pure water, N/sodium chloride, and N/hydrochloric acid. In each case the percentage of swelling was water, 20.5, N/sodium chloride, 42.5, and N/hydrochloric acid, 60.5. Both salt and acid had been added to the well and examination of the basket, when removed, showed a swelling of approximately 45 per cent.

Concentrations at the bottom of the well showed that salt had been settling during the period 7th July through 3rd August (see Table V). On 1st August the chloride conductivity at the bottom was 1,600 and on 3rd August it was 20,500. During this interval no salt had appeared in the wells of the 5- or 10-foot zone.

TABLE V

Salt concentration in charging well before removal of basket

Date	SURFACE			Bottom		
	pH	Chloride	Conductivity	pH	Chloride	Conductivity
July						
7th	7.7	1.49	390			
11th	7.7	429.0	9,500			
14th	7.9	239.0	9,500			
18th	7.7	264.0	7,000			
20th	8.2	140.0	2,750			
21st	8.0	158.0	3,100			
24th	8.4	165.0	3,400			
26th	8.3	1,170.0	22,500			
27th	8.3	366.0	7,500			
28th	7.9	148.0	2,840			
29th	8.1	165.0	2,900			
August						
1st	8.1	25.5	860	8.3	72.8	1,600
3rd	7.5	165.0	3,600	7.3	1,095.0	20,500

The basket was not restored to the well and 5 kilograms of salt were added on 4th August and again on 9th August. Three pounds of hydrochloric acid were added on 8th August, 2 pounds on 29th August and another 2 pounds on 2nd

September Following the addition of salt on 9th August the chloride content of the well dropped rapidly to 28th August when the surface content was 29 and that of the bottom 37 (Table VI) The addition of acid brought the surface content up to 56 and the bottom content up to 151 A sample taken on 4th September showed surface content to be 95.7 and the bottom content 224

TABLE VI

Salt reaction in charging well during determination of direction of flow

Date	SURFACE			BOTTOM		
	pH	Chloride	Conductivity	pH	Chloride	Conductivity
August						
4th	7.5	280	4,850	7.5	280	4,900
4th*	+8.4	6,750	140,000	+8.4	6,500	136,000
5th	+8.4	1,775	38,200	+8.4	5,140	116,000
7th	+8.4	1,080	24,000	+8.4	1,970	31,500
8th	7.7	545	10,200	8.1	1,550	28,500
9th	6.7	330	6,200	6.3	1,018	19,500
11th		495	10,000		1,155	22,000
12th	7.7	330	6,000	7.5	1,145	20,750
14th	7.7	338	6,100	7.7	660	9,500
16th	8.1	280	4,600	8.1	404	7,000
18th	8.1	165	3,100	8.1	313	6,000
22nd	8.1	165	2,900	8.1	223	4,100
24th	8.1	147	2,600	7.9	214	3,950
28th	7.9	29	625	7.9	37	730
September						
1st	6.9	56	1,360	6.2	151	3,000
6th	6.9	90	1,500	6.5	151	2,850
8th	7.3	51	1,200	6.9	102	1,850

* With this exception, all samples were taken before addition of reagents

The charging well was thoroughly pumped out on 8th September and no more salt was added. As shown by Table VII the chloride content dropped very low and did not rise until 2 pounds of acid were added on 29th September. Samples taken on 2nd October showed a surface chloride content of 80.8, and a bottom content of 179.9. On 2nd October, one-half pound of fluorescein and one pound of potassium hydroxide were added.

TABLE VII

Salt reactions after pumping out charging well

Date	SURFACE			BOTTOM		
	pH	Chloride	Conductivity	pH	Chloride	Conductivity
September						
9th	7.5	6.9	480	7.5	24.4	800
11th	7.9	2.8	390	7.7	5.6	430
13th	8.3	5.3	400	8.4	4.9	390
15th	8.4	5.1	365	8.1	5.4	420
18th	8.4	4.9	340	8.1	6.1	425
20th	8.4	5.4	350	8.1	5.6	390
22nd	8.1	4.9	335	8.4	5.9	390
25th	8.4	5.5	345	8.1	5.5	400
29th	8.4	5.4	300	7.9	4.9	395
October						
2nd	6.5	80.8	1,850	6.3	179.9	3,100

Receiving wells 2, 5 and 7 of the 10-foot zone had the tops of the well screens 17 feet 10 inches below the ground surface. Well 5 proved to be in the centre of

the salt flow and wells 2 and 7 were at the margins, making a stream of about $7\frac{1}{2}$ feet in width. Salt first appeared in well 5 on 11th August, and was observed in wells 2 and 7 on 14th August. It rose in concentration continuously through 24th August and then began to recede (Table VIII)

TABLE VIII

Salt recovery in wells 2, 5 and 7

Date	CHARGING WELL BOTTOM		WELL 2		WELL 5		WELL 7	
	pH	Chloride	pH	Chloride	pH	Chloride	pH	Chloride
August								
9th	6.3	1,018.0	7.7	0.99	8.1	3.96	7.5	1.16
11th		1,155.0		0.99		74.5		3.96
12th	7.5	1,145.0	7.5	4.04	7.7	140.3	7.5	4.50
14th	7.7	660.0	7.5	10.0	7.7	148.5	7.5	10.8
15th	7.8	460.0	7.5	15.2	7.7	165.0	7.5	13.3
18th	8.1	313.0	7.5	28.0	7.7	181.5	7.5	20.0
24th	7.9	214.0	7.5	38.8	7.7	235.5	7.5	44.6
28th	7.9	37.5	7.5	20.9	7.7	222.7	7.5	32.4
September								
6th	6.5	151.0	7.5	29.7	7.6	198.0	7.5	44.8
8th	6.9	102.0	7.5	30.3	7.6	206.0	7.5	44.8

There was a second rise of chloride content in these wells from 15th to 18th September which may have been associated with the addition of acid to the charging well made on 29th August and 2nd September (Table IX). No rise in salt

content was observed in the wells of this zone following the addition of one pound of acid on 14th July. There was an apparent effect upon the salt concentration when 2 pounds of acid were added, while the addition of 3 pounds of acid (on 8th August) resulted in a sharp rise of the salt content in the 10-foot zone within 4 days (by 11th August).

TABLE IX

Salt recovery in wells 2, 5 and 7 after charging well had been pumped out

Date	CHARGING WELL BOTTOM		WELL 2		WELL 5		WELL 7	
	pH	Chloride	pH	Chloride	pH	Chloride	pH	Chloride
September								
9th	7.5	24.4	7.0	24.1	7.7	165.0	7.5	39.6
11th	7.7	5.0	7.5	28.4	7.9	165.0	7.5	34.3
13th	8.4	4.9	7.7	29.7	7.7	173.2	7.5	58.1
15th	8.1	5.4	7.5	32.3	7.7	200.0	7.5	57.6
18th	8.1	6.1	7.5	40.3	7.7	165.0	7.3	60.1
25th	8.1	5.5	7.5	10.8	7.5	165.0	7.5	25.1
29th	7.9	4.9	7.5	21.7	7.7	107.5	7.5	19.1
October								
2nd	6.3	179.9	7.5	17.8	7.5	148.5	7.3	10.8
5th			7.5	10.5	7.5	67.5	7.5	4.6
9th			7.7	10.5	7.5	42.9	7.5	3.3

It is well known that salt is used to put a glaze on spun concrete pipe, but the amount is too small to account for the results indicated in Table IX, so such objections may be discounted.

By 13th October the charging well had returned almost to normal and the conductivity at surface and bottom was 430 (Table X) It was decided therefore to make the determination for velocity For this purpose 25 pounds of salt were added on 16th October, 2 pounds of hydrochloric acid on 20th October, and 2 pounds of hydrochloric acid on 28th October As in the other experiments, there was an appreciable rise in the chloride content after the addition of the acid

TABLE X

Salt reactions in charging well during determinations for velocity

Date	SURFACE			BOTTOM		
	pH	Chloride	Conductivity	pH	Chloride	Conductivity
October						
13th			430			430
17th			3,400			26,000
18th	7.7		2,300	7.7		7,200
20th		117.9	1,400		238.3	2,300
21st	3.8	491.6	5,500	3.8	567.6	5,500
23rd	6.3	212.2	1,950	5.3	498.3	4,100
29th	4.9	412.5	3,100	5.1	567.6	4,500
31st	6.2	303.6	2,900	6.0	412.5	4,100
November						
2nd	6.7	214.5	2,150	6.5	363.0	3,250
4th	7.5	16.5	525	7.3	33.0	680

Less salt was added in the experiment for velocity and, as appears in Table X, there was less reaction in the charging well As indicated in

Table XI, the reaction in the receiving wells was also less and of shorter duration

TABLE XI
Salt recovery in wells 2, 5 and 7

Date	CHARGING WELL BOTTOM		WELL 2		WELL 5		WELL 7	
	pH	Chloride	pH	Chloride	pH	Chloride	pH	Chloride
October								
13th			7.9	10.5	7.7	35.6	7.5	1.3
20th		117.9	7.7	9.9	7.5	26.0	7.5	3.3
21st	3.8	567.0	7.7	13.5	7.7	27.0	7.5	3.0
23rd	5.3	498.3	7.6	11.4	7.5	32.6	7.5	3.9
26th			7.7	12.2	7.5	33.6	7.5	1.9
28th	5.1	567.6	7.5	11.8	7.7	38.6	7.4	2.6
30th	5.1	551.3	7.5	12.0	7.5	42.2	7.4	1.9
31st	6.0	412.5	7.5	10.2	7.6	39.5		
November								
2nd	6.5	214.5	7.5	11.8	7.5	45.5	7.8	2.9
4th	7.3	33.0	7.6	9.5	7.5	38.2	7.5	1.3

This study indicates that salt is probably a poor instrument for the detection of direction and velocity of ground water flow in an alkaline soil because it accumulates in the charging well and must be dislodged with acid before it can be forced into the stream. Fluorescein also proved to be useless inasmuch as what was added to the charging well on 2nd October did not appear in the wells of the 10-foot zone until 3rd January, a full 3 months later. When tapped in the 10-foot wells

the salt was found in the stratum containing kankar. It will be recalled that the 5-foot wells did not enter this stratum which may explain the fact that salt was never recovered.

SUMMARY

When sodium chloride is used as an indicator for soil water flow in an *alkaline* soil, the salt flows from the charging well downward and, doubtless to some extent, laterally. Therefore, it cannot be considered a satisfactory medium for the measurement of velocity and it may also be an imperfect indicator for direction of flow. This conclusion applies in particular to alluvium soils and to a greater extent to quicksand soils.

Likewise, fluorescein made up in an alkaline solution was proved unsuitable for an alkaline soil, for in the present experiment fluorescein was added to the charging well on 2nd October and appeared in the wells of the 10-foot zone on 3rd January, a full 3 months later.

Salt appeared in the receiving wells only after the addition of acid to the charging well. Salt appeared in the deep wells which tapped the area containing kankar, and never appeared in the shallow or medium deep wells which did *not* tap the stratum containing kankar.

ACKNOWLEDGMENTS

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REFERENCE

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HÆMATOLOGICAL STUDIES IN INDIANS

Part XIV

THE MEASUREMENT OF THE RED CELL DIAMETER STANDARD PRICE-JONES' CURVE FOR AN INDIAN POPULATION

BY

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The methods in common use—There are at present three principal methods for measurement of the diameter of red cells, namely —

- (2) The images of the cells at a fixed magnification (usually 1,000) are projected on to a sheet of paper, and the outlines of the images accurately traced, these are measured with the help of a diagonal scale calibrated to give the size in accordance with the fixed magnification. Alternatively, the outlines of the cells can be drawn on a piece of paper by means of a camera lucida.
- (ii) Hynes and Martan (1936) have described a more rapid method of measurement. The images of the cells are projected on to a ground-glass screen at a fixed magnification (usually 2,000 times), and these are directly measured on the screen by superimposing a celluloid protractor, on which circles with diameters varying by the equivalent of 0.25μ have been drawn with a fine pair of dividers.
- (iii) Measurement with the help of a special micrometer ocular. The special ocular can be placed in the draw-tube of any microscope. As one

looks through it, the vertical scale which overlies any object brought into focus can be seen. The measurements on this scale can be calibrated by means of a stage micrometer for the particular tube length and lenses employed. By means of a micrometer screw and calibrated drum-head, the vertical scale can be moved from side to side and thereby measurements made in a horizontal as well as in a vertical direction.

The first method was originally used by Price-Jones and this was the method adopted by Napier and Das Gupta (1936). The alternative of using a camera lucida was utilized by Maplestone (Chaudhuri, 1933). The method of Hynes and Martin was modified by Sankaran and Rao (1938),* and adopted by the latter in their haematological studies. In the original method of Hynes and Martin, the images of the red cells are projected on a *horizontal* ground-glass at a fixed magnification of 2,000, by means of a microscope and a pointolite of 100 c.p. Sankaran and Rao (*loc cit*) employed a Bausch and Lomb euscope so that the images of cells were projected on the *vertical* ground-glass screen of the instrument and the source of illumination was a powerful carbon arc lamp working at 220 volts and 4.5 amperes. They used higher magnifications, and the series of circles on the celluloid protractor scale used were graded to correspond to intervals of 0.5μ . The instrument actually in use at the All-India Institute of Hygiene & Public Health, Calcutta, had a magnification of about 4,200, a Leitz microscope with $1/12$ objective, 170 mm tube length and $28\times$ ocular being used. The series of circles on the celluloid protractor increased by steps corresponding to 0.25μ .

Accuracy of methods—Regarding the accuracy of the different methods as compared with the classical method of Price-Jones, Whitby and Britton (1939) are of opinion that the method of using a camera lucida to draw the outlines of the cells is quicker but less accurate, and the results obtained by Price-Jones' and Hynes and Martin's methods are quite comparable, within acknowledged limits of experimental error, except when there is an extreme degree of poikilocytosis. The measurements by the special ocular micrometer are regarded by Musser and Wintrobe (1933) as being quite easy to make, although such measurements are not as accurate as are those obtained by the use of more complex methods.

Whilst using the method of Sankaran and Rao, one of the writers noticed that the diameter of a cell measured at the centre of the field was markedly different from that found when the same cell was moved to and measured at the periphery of the ground-glass screen of the euscope, e.g. a cell which measured 9μ at the centre measured 11μ at the periphery of the screen. Even when the image of a cell was moved through 2 or 3 inches a distinctly different measurement was obtained. Also there was some distortion of the shape of the image of the cell as it was moved from the centre to the peripheral part of the screen.

* In their paper, Sankaran and Rao (1938) did not mention in detail the optical apparatus that they used, or the magnification that this produced. We have now ascertained that this was about 2,000, so that our criticism of the apparatus, as it was reassembled in Calcutta, would probably not apply to the apparatus used by Sankaran and Rao in their investigation in 1938. The probability is strengthened by the fact that the coefficients of variation reported in their paper compare very favourably with those in the present one.

In order to find out the cause of this anomaly in the measurement, a micrometer objective scale (the divisions $10\ \mu$ apart) was placed under the microscope and its image was focused on to the ground-glass screen of the euscope. It was found that the image of the scale on the screen was markedly distorted by spherical aberration, so that the images of the parallel lines of the scale were not straight. By using the celluloid protractor, used for measurement with this instrument, it was found that the distance between the lines was $9.25\ \mu$ at the centre and $11.25\ \mu$ at the periphery and $10\ \mu$ about midway between the centre and the periphery.

It was thus decided that there was too much spherical aberration present to allow uniform measurement of a cell at different parts of the field. The high magnification (about 4,200) which necessitated the use of an eye-piece containing very thick lenses was believed to be the cause of this degree of spherical aberration.

In order to see if the defect due to spherical aberration could be eliminated, we reduced the magnification, and used a fixed magnification of 2,000, as in Hynes and Martin's original technique. By using a Zeiss microscope with an oil-immersion objective 90, tube length 154 mm and a Leitz eye-piece $15\times$, it was found that, with the particular euscope, a magnification of 2,000 could be attained. This was estimated as follows: the Zeiss stage micrometer scale was placed under the oil-immersion objective, and its image was focused on to the ground-glass screen of the euscope. By using a series of eye-pieces, it was found that with Leitz $15\times$ eye-piece and the tube length of 154 mm, the distance between the images of two consecutive lines was just 2 cm, i.e. the image of $10\ \mu$ was magnified to 2 cm, or $20,000\ \mu$, that is, 2,000 times. At this magnification there was very little spherical aberration, and in an area 4 inches by 3 inches about the centre of the field there was no spherical aberration at all. This was seen from the fact that the distance between the images of the lines measured 2 cm at every part of this reduced field. The red cells that could be included in this field were numerous to allow a rapid measurement of 500 cells.

A celluloid protractor with a series of circles corresponding to cell sizes between $4\ \mu$ and $12\ \mu$ diameter in $0.25\ \mu$ stages was prepared. At a magnification of 2,000, $0.25\ \mu$ equals 0.5 mm. The radii of the circles were thus made to increase by 0.25 mm. To obtain this small measurement, a diagonal scale was prepared which gave measurements of multiples of 0.25 mm. With the help of this diagonal scale circles were drawn in Indian ink on a celluloid sheet, with a pair of fine bow compasses, of diameters from 8 mm to 24 mm at 0.5 mm intervals, which corresponded to cells of diameters from $4\ \mu$ to $12\ \mu$. Circles corresponding to smaller and larger measurements were drawn on another sheet.

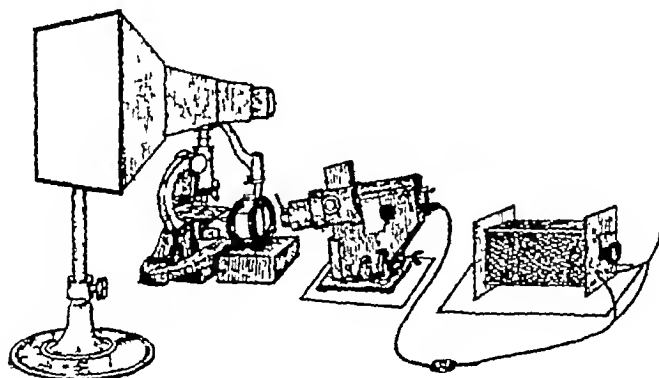
DETAILS OF TECHNIQUE

Apparatus required

- (i) A Bausch and Lomb euscope with projection screen
- (ii) A mechanical feed arc lamp with condenser, working at 4.5 amperes and 220 volts
- (iii) A glass container filled with distilled water for cooling the beam of light from the arc lamp (filter)

- (iv) A microscope with oil immersion objective and eye piece to give a magnification of 2,000 on the projection screen of the euscope
- (v) A stage micrometer scale (Zeiss) with divisions $10\ \mu$ apart
- (vi) A celluloid cm/mm scale
- (vii) Celluloid protractor for measuring the cells (*vide supra*)

Assembling and adjusting the apparatus—The Text-figure shows the euscope with microscope and arc lamp assembled. The concentrated beam of light from the arc lamp, cooled by passage through water in the filter, is focused on to the concave mirror of the microscope which reflects it through the condenser, the objective and the eye-piece of the microscope. The total reflexion prism of the euscope placed over the ocular reflects the light on to the opaque screen of the euscope. The condenser is fully raised up and, with the high power objective turned on, a bright beam of light is focused on the opaque screen. After the illumination has been adjusted, the micrometer scale is placed under the objective of the microscope and the image of the scale is focused on the opaque screen, first is used the low power and finally the oil-immersion objective. A final adjustment of light is made to give the maximum illumination. The opaque screen is now moved off and the image is focused on to the ground-glass projection screen of the euscope. By moving the microscope and/or the prism, the image of the scale is so projected that the lines of the scale are vertical and parallel and entirely free from spherical aberration, at least within an area of about 4 inches by 3 inches marked out around the centre of the viewing screen.



TEXT-FIGURE

The magnification of the apparatus is adjusted to be 2,000 by the use of a suitable oil-immersion objective and eye-piece, and varying the length of the draw-tube of the microscope (*vide supra*).

Measuring the cells—The micrometer scale is next removed, the blood film, stained with a Romanowsky stain and counterstained with 1 per cent aqueous eosin solution, is placed under the oil-immersion objective, and the image of the cells is focused on the ground-glass screen of the euscope. Only those images of

the cells that fall within the reduced field are measured. The celluloid protractor with the series of graduated circles is superimposed on the image of the corpuscles to find the circle that fits the image of each cell. Each cell is measured to the nearest 0.25μ . The measurement of circular corpuscles is quite easy. In the case of the irregular-shaped corpuscles, a circle is found such that the area of the corpuscle falling outside the circle is about the same as the area of the circle unfilled by the image of the corpuscle. Five hundred cells are measured from different parts of the blood film. It is better to avoid the 'tail' end and the thick parts of the smear as in the former the cells are excessively spread out and in the latter the cells overlap and are not well spread.

Recording the results—By this method, 500 cells can be measured in about 40 to 90 minutes, the time depending on the size of the cells and the degree of the poikilocytosis, the larger and more irregular the cells the longer is the time required. An assistant is needed to note down the measurements and record the number of cells counted with a counting machine of the type of a Veeder counter.

From the figures obtained the mean diameter, standard deviation and coefficient of variation are calculated. A Price-Jones' curve is drawn by plotting the figures on graph paper. The degree of microcytosis and macrocytosis can also be determined from this curve by comparison with the maximum and minimum ideal curves, the overlapping of a curve beyond the minimum ideal curve on the low side and the maximum curve on the high side indicates the degree of microcytosis and macrocytosis, respectively.

Test for accuracy of the technique—For this, one single slide was examined repeatedly by the same observer. He made 9 separate trials, measuring 500 cells on each occasion. Table I gives the figures in detail and also the mean and standard deviation of the cell diameter for each trial. The analysis of variance gives the following results—

Test of significance for the difference between means in different trials

Source of variation	Degrees of freedom	Sum of squares	Mean square	Ratio of mean squares
Between trials	8	5.979	0.747	2.306
Within trials	4,491	1,455.400	0.324	
TOTAL	4,499	1,461.379		

TABLE I

	I	II	III	IV	V	VI	VII	VIII	IX
5 00	1		3	3	1				1
5 25	3	7	9	2	3	9	4	6	2
5 50	9	14	16	16	6	22	18	10	7
5 75	36	33	28	37	32	25	34	26	30
6 00	66	73	72	73	58	75	67	69	73
6 25	70	81	91	62	62	62	74	59	65
6 50	84	86	100	89	103	104	107	113	116
6 75	61	77	63	60	80	58	70	93	67
7 00	63	49	40	70	63	66	50	52	55
7 25	64	43	38	49	51	42	46	40	47
7 50	24	24	23	24	22	19	18	15	18
7 75	10	7	8	6	8	4	5	9	11
8 00	5	5	6	4	7	9	4	5	6
8 25	3	1	0	2	4	1	2	2	2
8 50	1		2	3		2	1	1	
8 75			0			2			
9 00			1						
Mean diameter	6 6085	6 5275	6 5025	6 5620	6 6190	6 5435	6 5245	6 5645	6 5750
Standard deviation	0 5898	0 5601	0 5784	0 5936	0 5552	0 6037	0 5516	0 5378	0 5440

The ratio between the mean squares is 2 306 For 5 per cent significance the ratio must be 1 96 and for 1 per cent 2 55 The ratio thus lies between these two values

It is thus clear that this process of estimating the mean cell diameter involves errors other than those due to random sampling, these are other errors of experimentation Variations in cell diameters due to all these errors will be taken into consideration if we assume $\sqrt{\frac{0.747}{500}} = 0.03865$ as the estimate of the standard error of the mean and not $\sqrt{\frac{0.324}{500}} = 0.02546$ Thus, in using the conventional limit of 3 times (say) the standard error of the mean for testing homogeneity, the

value 0.03865 should be adopted as the estimate of the standard error of the mean. This is equivalent to using $\frac{0.03865}{0.02546} \times 3 = 4.55$ times the estimate of the standard error of the mean, if the estimate had been made on the basis of the variation among the 500 cells observed at each trial. Thus, it appears that to allow for error of experimentation including that due to sampling, in judging significant differences in mean diameters, the smallest difference of any importance should be at least $3 \times 0.03865 \mu$, i.e. 0.12μ .

Test of significance for difference in standard deviation—The homogeneity of the standard deviation in the different trials was tested by means of Neyman and Pearson's L_1 test and no significant difference emerged.

Comparison of the results obtained by measurements at a magnification of about 4,200 with those obtained at a magnification of 2,000 times—A number of slides in which the mean diameter, standard deviation, etc. were obtained by measurements at a magnification of 4,200 were examined by the technique described in this paper. Table II gives the mean diameters and standard deviations obtained by the two methods. It will be seen that the mean diameters are practically the same in a number of cases. This is not surprising as the scale used is correct at a point about midway between the centre and the periphery of the ground-glass screen, the high measurements in the peripheral region cancelling the low measurements of the centrally placed cells—images of the cells over most of the (though not the entire) field having been measured. The standard deviation and the coefficient of variation in every instance are greater than those obtained by the present technique, the difference in most instances being very considerable. This means that the Price-Jones' curve will have a wider base. That there is greater probability of error in measurement in the former method is also evident from the optical defects enumerated above.

TABLE II

Slide number	MEAN CORPUSCULAR DIAMETER		STANDARD DEVIATION		COEFFICIENT OF VARIATION	
	By method I	By method II.	By method I	By method II	By method I	By method II
I	7.614	7.522	0.660	0.525	8.67	6.9
II	7.759	7.815	0.650	0.560	8.4	7.17
III	7.867	7.881	0.637	0.542	8.1	6.8
IV	7.834	7.842	0.645	0.530	8.2	6.79
V	7.300	7.44	0.680	0.415	8.9	5.5
VI	7.643	7.633	0.637	0.542	8.3	7.1
VII	7.250	7.150	0.637	0.630	9.48	8.8
VIII	7.817	7.745	0.667	0.650	8.5	8.3
IX	7.880	7.900	0.620	0.560	7.8	7.5
X	7.430	7.600	0.690	0.610	9.3	8.0

Method I = by using a magnification of about 4,200

Method II = by using a magnification of 2,000

Determination of the mean, standard deviation and coefficient of variation of red cell diameters in normal Indians (mainly Bengalees) Standard curve for normal Indians—The technique described above was adopted to determine the mean, standard deviation and coefficient of variation of cell diameters of a normal Indian population (mainly Bengalees) resident in Calcutta. Blood films were made from 25 persons (doctors and laboratory assistants) and these were stained with a Romanowsky stain and counterstained with aqueous eosin. In each case the blood was taken from the finger and was collected in the forenoon. Table III gives the mean, standard deviation and coefficient of variation of red blood cell diameter of these 25 normal men. It will be seen that the mean diameter obtained is 7.3438μ with a standard deviation of 0.1328μ .

TABLE III

Serial number	Mean cell diameter (MCD)	Standard deviation (σ)	Coefficient of variation (v)
1	7.316	0.520	7.11
2	7.479	0.535	7.15
3	7.283	0.451	6.10
4	7.257	0.488	6.72
5	7.455	0.445	5.98
6	7.394	0.482	6.52
7	7.374	0.528	7.16
8	7.288	0.490	6.72
9	7.315	0.350	4.78
10	7.431	0.525	7.06
11	7.381	0.565	7.65
12	7.449	0.548	7.36
13	7.196	0.475	6.60
14	7.395	0.520	7.03
15	7.487	0.462	6.17
16	7.404	0.530	7.07
17	7.440	0.498	6.69
18	7.431	0.423	5.69
19	7.290	0.517	7.09
20	7.378	0.501	6.79

TABLE III—*concl'd*

Serial number	Mean cell diameter (MCD)	Standard deviation (σ)	Coefficient of variation (v)
21	7.216	0.558	7.73
22	7.571	0.460	6.08
23	7.062	0.517	7.32
24	7.195	0.505	7.02
25	7.019	0.403	5.74
Mean	7.3438	0.4918	6.697
Standard deviation	0.1328	0.0493	0.667
Coefficient of variation	1.81 per cent	10.02 per cent	9.96 per cent
Standard deviation $\times \sqrt{\frac{25}{24}}$	0.1355	0.0503	0.6806

The analysis of variance to test differences in the means of the 25 persons is as follows —

Source of variation	Degrees of freedom	Sum of squares	Mean square	Ratio of mean squares
Between persons	24	220.32	9.180	37.47 (very significant)
Within persons	12.475	3054.05	0.245	
TOTAL	12,499	3274.37		

There is, thus, a significant difference between the means of the different persons. It has already been observed that experimental errors, other than the error due to sampling, also cause differences in means, and due to all these causes the estimate of the standard error of the mean can reasonably be increased to only $\frac{0.03865}{0.02546} = 1.52$ times, the estimate made on the basis of the variation between the 500 cells measured in each experiment. But the estimate of the standard error of the mean of the 25 individuals is equal to $\sqrt{\frac{9.180}{500}} = 0.1355$ whereas the estimate of the standard error based only on the variations between cell diameters

at each trial is $\sqrt{\frac{0.245}{500}} = 0.02214$. The ratio between these estimates is 6.12 which is considerably greater than 1.52. It follows that the variation in the means of the different individuals is very much more than can be accounted for by experimental errors only, and such heterogeneity in a group of 'normal' individuals has been observed by other workers. Therefore, the adoption of ± 3 times the standard deviation of the means of the 'normal' individuals, following Price-Jones, should give a range sufficiently wide to take into account all errors of experimentation, including that of random sampling. We have, however, adopted $\pm 3 \times \text{standard deviation} \times \sqrt{\frac{n}{n-1}}$ as the range, where n is the number of observations, since the standard deviation by itself does not provide an unbiased estimate of the variability in the general normal population.

The range for the mean is thus from 6.937μ to 7.750μ . The range for the standard deviation when similarly calculated is from 0.3409μ to 0.6427μ and that of the coefficient of variation from 4.66 per cent to 8.74 per cent.

The range of mean, standard deviation and coefficient of variation of cell diameters

Locality	Authority	Range of mean cell diameter	Range of standard deviation	Range of coefficient of variation
London	Price Jones (1933)	6.686 to 7.718	0.4 to 0.5	5.3 to 7.3
Calcutta	Writers	6.937 to 7.750	0.34 to 0.64	4.66 to 8.74

Construction of ideal 'normal' curves about the maximum and minimum mean diameters

As stated above the minimum and maximum mean diameters for healthy Indians (Bengalees) were taken to be 6.937μ and 7.750μ , respectively. For the standard deviation of the 'normal' curves about these means, we took 0.4948μ which was obtained from the following formulæ —

$$S.D. = \sqrt{\frac{\sum (Sd)^2}{K}} \times \sqrt{\frac{500}{499}} \mu$$

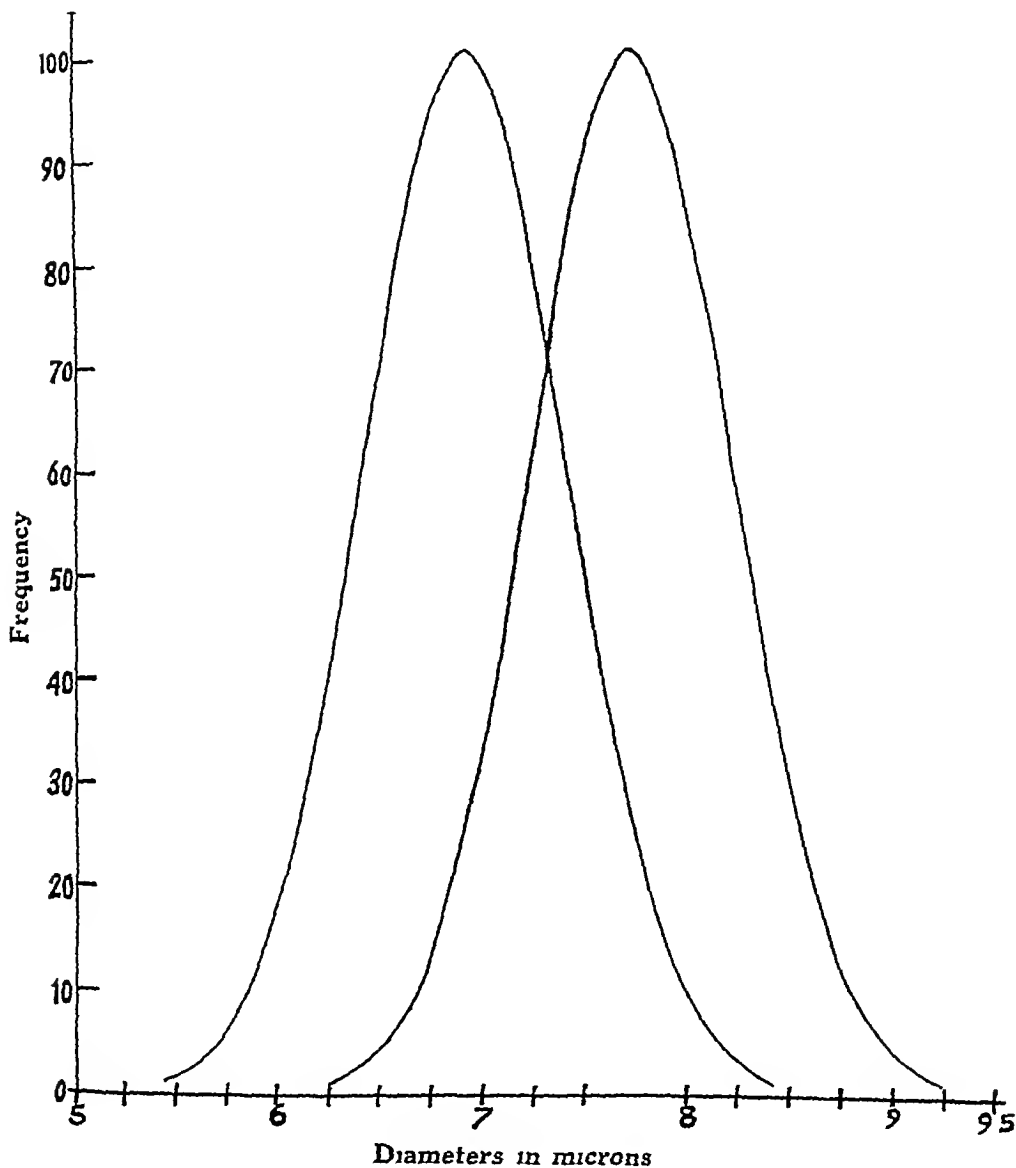
where $S.D.$ is the standard deviation of the 'normal' curve and Sd the standard deviation for the different individuals, and K the number of individuals

$$\begin{aligned} S.D. &= \sqrt{\frac{\sum (Sd)^2}{25}} \times \sqrt{1.002} \mu \\ &= \sqrt{\frac{6.1081}{25}} \times 1.001 \mu \\ &= 0.49429 \times 1.001 \mu = 0.4948 \mu \end{aligned}$$

The ordinates of the normal curves were calculated with the help of statistical tables and two normal curves were drawn about the maximum and minimum mean diameters. These curves cut at a point corresponding to the mean of the mean

GRAPH

Showing Price-Jones' curve based on Indian data



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INDEX OF AUTHORS

A

PAGE

- ACHARYA, B N See NIYOGI, S P
- ADAK, B See LAL, R B
- ADLER, S, and BER, M The Transmission of *Leishmania tropica* by the Bite of *Phlebotomus papatasi* (with 1 Plate) 803
- AGARWALA, S P See LAL, R B
- AHMED, I See SMITH, R O A
- AYKROYD, W R, and KRISHNAN, B G Rice Diets and Beriberi 551
- AYKROYD W R, and KRISHNAN, B G Infantile Mortality in the Beriberi Area of the Madras Presidency (with 1 Chart in text) 703

B

- BASAK, K C See LAL, R B, and SATYA SWAROOP
- BASAK, M N See BASU, K P
- BASU, K P, BASAK, M N, and DE, H N Studies in Human Nutrition Part III Protein, Calcium and Phosphorus Metabolism with Typical Indian Dietsaries 105
- BASU, N M, and DE, N K Assessment of Vitamin A Deficiency amongst Bengalees and Determination of the Minimal and Optimal Requirements of Vitamin A by a Simplified Method for Measuring Visual Adaptation in the Dark (with 5 Graphs in text) 591
- BASU, P N, and SEN, S N A Peptic Digest Broth for the Formation of *Clostridium tetani* Toxin 689
- BASU, U P See BOSE, A N
- BER, M See ADLER, S
- BHAGWAT, G A See TELANG, D M
- BHASKARA MENON, T See MENON, T BHASKARA
- BHAVE, P D Diet Surveys in the Central Provinces and Berar 99

	PAGE
BISWAS, R B <i>See</i> KIRWAN, E O'G	
BOSE, A N , DAS GUPTA, S J , and BASU, U P Activity of 2-Sulphamido-4-methylthiazole against Type I Pneumococcus Infections in Mice A Preliminary Note	265
BOSE, R <i>See</i> DHARMENDRA	
BRIAN R DYER <i>See</i> DYER, BRIAN R	
C	
CHAKRAVARTI, M <i>See</i> CHOPRA, R N	
CHANDRA, S N <i>See</i> GREVAL, S D S	
CHANDRA SEKAR, C <i>See</i> NAPIER, L EVERARD	
CHATTERJEE, S C <i>See</i> DE, S P	
CHATTERJEE, S K , and MITTER, K N Hæmolytic <i>Streptococci</i> in the Throat of apparently healthy Persons Its Incidence and Causal Relationship to Puerperal Infection	483
CHATTERJEE, S K , and MITTER, K N Significance of Hæmolytic <i>Streptococci</i> in Parturient Women (<i>with 1 Graph in text</i>)	491
CHATTERJI, S R <i>See</i> LAL, R B	
CHAUDHURI, D K <i>See</i> GHOSH, B N	
CHITRE, R G <i>See</i> NIYOGI, S P	
CHOPRA, G S <i>See</i> CHOPRA, R N	
CHOPRA, I C <i>See</i> SEHRA, K B	
CHOPPA, R N , CHOPRA, G S and ROY, A C Urinary Excretion of Morphine in Opium Addicts with and without Lecithin-Glucose Treatment	195
CHOPRA, R N , GANGULY, S C , and RAO, S SUNDAR Protein Fractions and other Physical Properties of Hydrocele Fluid	253
CHOPRA, R N , and CHAKRAVARTI M A Preliminary Note on the Pharmacological Action of the Alkaloids of <i>Rauwolfia serpentina</i>	763
CHOPRA, R N , GUPTA J C , and CHOPRA, G S Pharmacological Action of the Essential Oil of <i>Curcuma longa</i>	769
CHOPRA, R N <i>See</i> ROY, A C	

D

DAS, B C <i>See</i> GREVAL, S D S
DAS GUPTA, A C <i>See</i> LAL, R B

- DAS GUPTA C R *See* NAPIER L EVERARD
- DAS GUPTA, S J *See* BOST A N
- DATTA, N C Metallic Contamination of Foodstuffs Part IV Investigations on Contamination of Foodstuffs with Copper from Brass and Bronze Vessels and the Metabolic Experiments on the Absorption and Excretion of Copper in Rats 751
- DATTA, S K *See* DE, S P
- DE, H N *See* BASU, K P
- DE, N K *See* BASU N M
- DE, S P, DATTA, S K and CHATTERJEE, S C Bacteriostatic Effect of Human Bile after Oral Administration of Hexamine and Sulphanilamide as studied in a Patient with Biliary Fistula 271
- DE, S S *See* GHOSH B N
- DEY, N C *See* MAPLESTONE P A
- DHARMENDRA Complement-Fixation by Leprous Sera after Absorption by various Acid-fast Bacilli 523
- DHARMENDRA and BOSE, R Complement-Fixation in Leprosy with Antigens prepared from various Acid-fast Bacilli 7
- DHURANDHAR, C B The Reliability of Guinea-pig Inoculation Test for the Diagnosis of Human Tubercular Affections (*with 1 Chart in text*) 531
- DOGRA, J R Studies on Peptic Ulcer in South India Part III Experimental Production of Gastro-duodenal Ulcer 311
- DOGRA J R Studies on Peptic Ulcer in South India Part IV Incidence of Peptic Ulcer in India with Particular Reference to South India (*with 1 Map in text*) 665
- DYER, BRIAN R Studies of Ground Water Pollution in an Alkaline Alluvium Soil (*with 1 Map, 3 Graphs and 1 Diagram in text, and 1 Plate*) 867
- DYER, BRIAN R Sodium Chloride as an Indicator for the Determination of the Flow of Soil Water 891

E

- EVERARD NAPIER, L *See* NAPIER L EVERARD

F

- FABISCH WALTER and HAMBURGER, H J Observations on the Physical Development of Punjabi Boys (*with 6 Graphs in text*) 613
- FRENKEL, J Note on an Intracellular Stage of *Leishmania chameleons*, Wenzon 1921 (*with 2 Text-figure*) 811

G

- GANGULY, S C See CHOPRA, R N
- GHOSE, R, and MUKERJI, B Differences in the Rate of Chloral Clearance in Blood in Normal and Liver-damaged Dogs (*with 2 Graphs in text*) 639
- GHOSH B N, DE, S S and CHAUDHURI, D K Separation of the Neurotoxin from the Crude Cobra Venom and Study of the Action of a Number of Reducing Agents on it 367
- GHOSH, L M, and MAPLESTONE, P A An Improved Method of Growing Pure Cultures of Ringworm Fungi (*with 1 Plate*) 691
- GIRI, K V, and NAGANNA, B An Adsorption Method for the Estimation of Nicotinic Acid Content of Foodstuffs 125
- GIRI, K V, and NAGANNA, B An Adsorption Method for the Estimation of Nicotinic Acid Content of Animal Tissues and Blood 585
- GIRI, K V See KRISHNAMURTHY, P V
- GOKHALE, S K Blood Urea Clearance in Normal Indians A Study based on the Examination of 110 Normal Indian Men 627
- GREVAL, S D S, DAS, B C, and SEN GUPTA, P C Preparation and Use of the Witebsky, Klingenstein and Kuhn (W K K) Antigen 527
- GREVAL, S D S, and CHANDRA, S N An Important Antigenic Difference between Hæmagglutinogens M and N 547
- GREVAL, S D S, CHANDRA, S N, and DAS, B C Complement-Fixation in Hydatid Disease Suggestions 203
- GREVAL, S D S, CHANDRA, S N, and WOODHEAD, L S F On Isohæmagglutination Nomenclature, Titration of Isohæmagglutinins, Need for Revision of Technique of Grouping Blood, etc (*with 2 Plates*) 231
- GUPTA, J C See CHOPRA, R N

H

- HALDER, K C See SMITH, R O A
- HAMBURGER, H J See FABISCH, WALTER

I

- IYENGAR, M O T Occurrence of *Wuchereria bancrofti* Infection in a Rural Area 677
- IYENGAR, N K Competition of Protein Substrates towards Proteolytic Enzymes 655

J

- JOB, T J Food and Feeding Habits of the Glassfishes (*Ambassis* Cuv & Val) and their Bearing on the Biological Control of Guinea-worm and Malaria (*with 2 Text-figures*) 851

K

- KIRWAN, E O'G, SEN, K, and BISWAS, R B Nutrition and its Bearing on Preventable Blindness and Eye Diseases in Bengal Preliminary Report 119
- KOCHHAR, B D Nicotinic Acid in Blood 133
- KOCHHAR, B D Nicotinic Acid in Blood and in Urine 341
- KRISHNAMURTHY, P V, and GIRI, K V Further Studies on the Influence of Pyrophosphate on the Oxidation of Vitamin C (*with 2 Graphs in text*) 71
- KRISHNAN, B G See AYKROYD, W R
- KRISHNAN, K V, and NARAYANAN, E K Preparation of Peptone for Bacteriological Work (*with 2 Text-figures*) 541
- KRISHNASWAMI, A K See VENKATRAMAN, K V -

L

- LAHIRI, M N On the Foetal Infection by *L. icterohæmorrhagæ* in a Rat 685
- LAL, R B, and DAS GUPTA, A C Investigations into the Epidemiology of Epidemic Dropsy Part X A Note on an Outbreak of Epidemic Dropsy associated with the Use of Mustard Oil pressed from Seeds adulterated with Seeds of *Argemone mexicana* (*with 1 Map in text and 1 Plate*) 157
- LAL, R B, CHATTERJI, S R, AGARWALA, S P, and DAS GUPTA, A C Investigations into the Epidemiology of Epidemic Dropsy Part XI Biological Test of Specific Toxin in Samples of Oil (*with 11 Graphs in text and 3 Plates*) 167
- LAL, R B, DAS GUPTA, A C, AGARWALA, S P, and ADAK, B Investigations into the Epidemiology of Epidemic Dropsy Part XIII Application of the Biological Test to Modified Argemone Oil and its Derivatives (*with 16-Graphs in text*) 813
- LAL, R B, DAS GUPTA, A C, MUKHERJI, S P, and ADAK, B Investigations into the Epidemiology of Epidemic Dropsy Part XIV Feeding Experiments on Human Subjects to test the Toxicity of some of the Derivatives and Modifications of Argemone Oil 839

	PAGE
LAL, R B, RAJA, K C K E, and SATYA SWAROOP Statistical Inquiry into the Epidemiology of Cholera in Bengal Part I A General Review of the Epidemiological Features of Cholera in Different Parts of Bengal (<i>with 4 Maps in text</i>)	425
LAL, R B, RAJA, K C K E, SATYA SWAROOP, and BASAK, K C Statistical Inquiry into the Epidemiology of Cholera in Bengal Part II Formation of Homogeneous Cholera Districts (<i>with 8 Maps in text</i>)	441
LAL, R B See MUKHERJI, S P	
M	
MAJUMDAR, B N The Vitamin A Content of some Indian Fish-Liver Oils	95
MAPLESTONE, P A, and DEV, N C Further Laboratory Tests on the Fungistatic and Fungicidal Effects of various Substances	23
MAPLESTONE, P A See GHOSH, L M	
MATHUR, K B L See MUKHERJI, S P	
MENON, K P <i>Staphylococci</i> in Vaccine Lymph	259
MENON, T BHASKARA, and RAMAMURTI, B The Behaviour of the Infective Larvæ of <i>Wuchereria bancrofti</i> with Special Reference to their Mode of Escape and Penetration of Skin (<i>with 1 Plate</i>)	393
MITRA, K Dietary and Physique of Mining Population in Jharia Coal Fields (Bihar)	143
MITRA, K, and MITTRA, H C Estimation of the Proximate Principles of Food in a few Edibles by Chemical Methods	315
MITTER, K N See CHATTERJEE, S K	
MITTRA, H C See MITRA, K	
MUKERJI, B See GHOSE, R, and SEHRA, K B	
MUKHERJI, S P, LAL, R B, and MATHUR, K B L Investigations into the Epidemiology of Epidemic Dropsy Part XII Isolation of Active Substances from Toxic Oils (<i>with 1 Plate</i>)	361
MUKHERJI, S P See LAL, R B	

N

NAGANNA, B See GIRI, K V	
NAPIER, L EVERARD, NEAL-EDWARDS, M I, and DAS GUPTA, C R Haematological Studies in Indians Part XIII Normal Indian Women in Calcutta (<i>with 1 Graph in text</i>)	375

	PAGE
NAPIER, L EVERARD, SEN GUPTA, P C, and CHANDRA SEKAR, C Hæmatological Studies in Indians Part XIV The Measurement of the Red Cell Diameter Standard Price-Jones' Curve for an Indian Population (<i>with 1 Text-figure and 1 Graph in text</i>)	903
NARAYANAN, E K Some Observations on the Preparation of Mannose	1
NARAYANAN, E K See KRISHNAN, K V	
NEAL-EDWARDS, M I See NAPIER, L EVERARD	
NIYOGI, S P, PATWARDHAN, V N, ACHARYA, B N, and CHITRE, R G Balanced Diets Part II Studies on the Nutritive Value of Fish	279
NIYOGI, S P, PATWARDHAN, V N, and SIRSAT, M V Studies on Basal Metabolism in Bombay Part III An Examination of the Factors Influencing the Basal Metabolism (<i>with 1 Graph in text</i>)	287

P

PANDIT, S R See READ, W D B	
PASSMORE, R, and SUNDARARAJAN, A R The Vitamin B ₁ Content of the Millets <i>Eleusine coracana</i> and <i>Sorghum vulgare</i> Whole Wheat grown under Different Manurial Conditions, and Rice Stored Underground	89
PATWARDHAN, V N See NIYOGI, S P	

R

RADHAKRISHNA RAO, M V See RAO, M V RADHAKRISHNA	
RAGHVENDER RAO, S See RAO, S RAGHVENDER	
RAHMAN, S A Alterations in the Electrocardiographic Features brought about by Digitalis (<i>with 1 Plate</i>)	659
RAHMAN, S A, and ZAIDI, M A Study on the Normal Polynuclear (Arneth) Count at Hyderabad-Deccan (<i>with 2 Graphs in text</i>)	225
RAJA, K C K E See LAL, R B, and SATYA SWAROOP	
RAJAGOPAL, K Dark-Adaptation Tests in Cases of Clinical Night-Blindness (<i>with 1 Diagram and 3 Graphs in text</i>)	351
RAJAGOPAL, K Physical and Chemical Methods of Estimating Vitamin A in Shark and Saw-fish Liver Oils	575
RAMAKRISHNAN, C S See VENKATRAMAN, K V	
RAMAMURTI, B See MENON, T BHASKARA	

	PAGE
RANGANATHAN, S Calcium Intake and Fluorine Poisoning in Rats	693
RANGANATHAN, S The Vitamin D Content of some Fish Oils	699
RAO, M V RADHAKRISHNA Pathological Changes occurring in the Parathyroids in Rats fed on a Poor Rice Diet (<i>with 3 Plates</i>)	137
RAO, S RAGHVENDER Rat-Fleas of Calcutta Investigated from a Point of View of Epidemiology of Plague (<i>with 1 Graph in text</i>)	51
RAO, S SUNDAR, and SUKHATME, P V Seasonal Variations in the Incidence of Filarial Lymphangitis (<i>with 11 Graphs in text</i>)	209
RAO, S SUNDAR See CHOPRA, R N	
READ, W D B, and PANDIT, S R Distribution of <i>V cholerae</i> and El Tor Type Strains in certain Rural Areas in India (<i>with 2 Diagrams in text</i>)	403
ROY, A C, and CHOPRA, R N Lecithin and Hæmolysis	773
ROY, A C See CHOPRA, R N	
ROY, D N See STRICKLAND, C	

S

SATYA SWAROOP, RAJA, K C K E, LAL, R B, and BASAK, K C Statis- tical Inquiry into the Epidemiology of Cholera in Bengal Part III Endemicity and Epidemicity of the Homogeneous Cholera Districts (<i>with 1 Map in text</i>)	465
SATYA SWAROOP A Modification of the Routine Dilution Tests and Tables showing the Most Probable Number of Organisms and the Standard Error of this Number	499
SATYA SWAROOP A Consideration of the Accuracy of Estimation of the Most Probable Number of Organisms by Dilution Test (<i>with 2 Graphs in text</i>)	511
SATYA SWAROOP See LAL, R B	
SEHRA, K B, CHOPRA, I C, and MUKERJI, B Experimental Liver and Biliary Damage and Serum Phosphatase (<i>with 3 Graphs in text</i>)	647
SEKAR, C CHANDRA See CHANDRA SEKAR, C	
SEN, K See KIRWAN, E O'G	
SEN, S N See BASU, P N	
SEN GUPTA, P C See GREVAL, S D S, and NAPIER, L EVERARD	
SHOURIE, K L Dental Caries in Indian Children	709
SIRSAT, M V See NIYOGI, S P	

	PAGE
SMITH, R O A , HALDER, K C , and AHMED, I Further Investigations on the Transmission of Kala-azar Part IV The Duration of Life and other Observations on 'Blocked' Flies (<i>with 2 Text-figures</i>)	783
SMITH, R O A , and AHMED, I Further Investigations on the Transmission of Kala-azar Part V An Inquiry into the Relation between Malaria and Kala-azar in a Rural Area (<i>with 1 Graph in text</i>)	789
SMITH, R O A , HALDER, K C , and AHMED, I Further Investigations on the Transmission of Kala-azar Part VI A Second Series of Transmissions of <i>L donovani</i> by <i>P argentipes</i>	799
STRICKLAND, C , and ROY, D N Myiasis-producing Diptera in Man	863
SUKHATME, P V See RAO, S SUNDAR	
SUNDARARAJAN, A R The Vitamin B ₁ Content of Human Milk	567
SUNDARARAJAN, A R See PASSMORE, R	
SUNDAR RAO, S See RAO, S SUNDAR	
SWAMINATHAN, M The Effect of Washing and Cooking on the Nicotinic Acid Content of Raw and Parboiled Rice	83
SWAMINATHAN, M Further Studies on the Cyanogen Bromide Method of Estimating Nicotinic Acid in Biological Materials (<i>with 1 Graph in text</i>)	325
SWAMINATHAN, M Urinary Excretion of Vitamin B ₆ by Rats	557
SWAMINATHAN, M A Method for the Estimation of Vitamin B ₆ in Urine	561
SWAROOP, SATYA See SATYA SWAROOP	

T

TELANG, D M , and BHAGWAT, G A Studies in the Vital Capacity of Bombay Medical Students Part I Statistical Correlation with Physical Measurements (<i>with 25 Graphs in text</i>)	723
---	-----

V

VEERARAGHAVAN, N Elimination of Excess Nerve Tissue from Antirabic Vaccine	303
VENKATRAMAN, K V , KRISHNASWAMI, A K , and RAMAKRISHNAN, C S Occurrence of <i>Vibrio El Tor</i> in Natural Sources of Water in the Absence of Cholera	419
VENKATRAMAN, K. V , and RAMAKRISHNAN, C S A Preserving Medium for the Transmission of Specimens for the Isolation of <i>Vibrio cholerae</i>	681

Index of Authors

	W	
WALTER FABISCH	<i>See</i> FABISCH, WALTER	PAGE
WOODHEAD, L S F	<i>See</i> GREVAL, S D S	
	Z	
ZAIDI, M A	<i>See</i> RAHMAN, S A	

INDEX OF SUBJECTS

- ABSORPTION, *see* acid fast bacilli, copper
 ACID, *see* nicotinic
 ACID FAST BACILLI complement-fixation
 by leprous sera after absorption by, 523, in
 leprosy with antigens from, 7
 ACTIVE SUBSTANCES, isolation of, from toxic
 oils, 361
 ADAPTATION, *see* dark adaptation
 ADDICTS, *see* opium
 ADSORPTION method for estimating nicotine
 acid content, of animal tissues and blood, 585,
 of foodstuffs, 125
 ALKALINE ALLUVIUM SOIL, ground water
 pollution in, 867
 ALKALOIDS of *Rauwolfia serpentina*, pharma-
 cology of, 763
 ALLUVIUM SOIL, *see* alkaline.
 AMBASSIS Cuv and Val, *see* glassfishes
 ANIMAL TISSUES, nicotine acid content of,
 585
 ANTIGEN preparation and use of W K K.,
 527, prepared from acid fast bacilli,
 complement-fixation in leprosy with, 7
 ANTIGENIC DIFFERENCE between
 haemagglutinogens M and N, 547
 ANTIRABIC vaccine elimination of excess
 nerve tissue from, 303
 ARGEMONE MEXICANA, *see* epidemic
 dropsy
 ARGENTIPES *Phlebotomus*, transmission of
 L. donovani by, 799

 BACILLI, *see* acid fast.
 BACTERIOLOGICAL work, preparation of
 peptone for, 541
 BACTERIOSTATIC effect of human bile, 271
 BALANCED DIETS, 279
 BANCROFTI, *Wuchereria* infective larvae of,
 393, in a rural area, 677
 BASAL METABOLISM in Bombay, 287
 BENGAL blindness in, 119, cholera in, 425,
 441, 465
 BENGALLEES, vitamin A deficiency in, 591
 BERAR, diet surveys in C P and, 99

 BERIBERI infantile mortality from, in
 Madras, 703 rice diets and, 551
 BIHAR, *see* Jharia coal fields
 BILIARY damage, experimental, 647,
 fistula, 271
 BIOLOGICAL control of guinea worm and
 malaria by glassfishes, 851, materials,
 cyanogen bromide method of estimating
 nicotinic acid in, 325, test of oils, 167, 813
 BLINDNESS nutrition and its bearing on, in
 Bengal, 119, clinical night-blindness, dark-
 adaptation in, 351
 'BLOCKED' FLIES, *see* kala azar
 BLOOD chloral clearance in, 639, nicotine
 acid content of, 133, 341, 585, technique of
 grouping, 231, urea clearance, 627
 BOMBAY basal metabolism in, 287, vital
 capacity in, 723
 BOYS, Punjabi, physical development of, 813
 BRASS vessels, contamination of foodstuffs
 with copper from, 751
 BROMIDE, *see* cyanogen
 BRONZE vessels, contamination of foodstuffs
 with copper from, 751
 BROTH, *see* peptic digest

 CALCIUM intake in rats, 693, metabolism,
 105
 CALCUTTA haematological studies in normal
 Indian women in, 375, rat fleas of, 51
 CAPACITY, *see* vital capacity
 CARDIOGRAPH, *see* electrocardiograph
 CARRIES, dental, in Indian children, 709
 CELLULAR, *see* intracellular
 CENTRAL PROVINCES, diet surveys in, 99
 CHAJELEONIS, *Leishmania*, intracellular
 stage of, 811
 CHEMICAL METHODS estimation of
 proximate principles of food by, 315 vitamin
 A in shark and saw fish liver oils by, 575
 CHILDREN, dental carries in Indian, 709
 CHLORAL CLEARANCE in blood in normal
 and liver damaged dogs, 639
 CHLORIDE, *see* sodium.

- CHOLERA** epidemiology of, in Bengal, 425, 441, 465, vibrio El Tor in water in absence of, 419
- CHOLERÆ**, *Vibrio* distribution of, in rural areas in India, 403, preserving medium for transmission of, 681
- CLEARANCE**, see blood urea, chloral
- CLOSTRIDIUM TETANI** toxin, peptic broth for, 689
- COAL FIELDS** (Jharia), dietary and physique of mining population in, 143
- COBRA** venom (crude), separation of neuro toxin from, 367
- COMPLEMENT FIXATION** in hydatid disease, 203, by leprosy sera after absorption by various acid fast bacilli, 523, in leprosy with antigens from acid fast bacilli, 7
- CONTAMINATION**, see metallic contamination, copper
- COPPER**, contamination of foodstuffs with, from brass and bronze vessels, 751, metabolic experiments on absorption and excretion of, in rats, 751
- CORACANA**, *Eleusine* (millets), vitamin B₁ content of, 89
- CORRELATION**, see vital capacity
- CULTURES** of ringworm fungi, method of growing, 691
- CURCUMA LONGA**, pharmacology of essential oil of, 769
- CURVE**, see Price Jones'
- CYANOGEN BROMIDE** method of estimating nicotinic acid, 325
- DAMAGE** see biliary, liver
- DARK-ADAPTATION** measuring visual, 591, in night blindness, 351
- DECCAN** (Hyderabad), normal polynuclear count at, 225
- DEFICIENCY**, see vitamin
- DENTAL CARIES** in Indian children, 709
- DIET** balanced, 279, poor rice, pathological changes in parathyroids in rats fed on, 137, rice and beriberi, 551, surveys in C P and Berar, 99
- DIETARY** and physique of mining population in Jharia coal fields, 143, protein, calcium and phosphorus metabolism with typical Indian, 105
- DIGEST**, see peptic digest broth
- DILUTION TEST**, 499, 511
- DIGITALIS**, alterations in electrocardiographic features by, 659
- DIPTERA** (myiasis producing) in man, 863
- DOGS**, chloral clearance in blood in normal and liver damaged, 639
- DONOVANI**, *Leishmania*, transmission of, by *P. argentipes*, 799
- DROPSY** (epidemic) epidemiology of, 157, 167, 361, 813, 830, outbreak of, associated with use of mustard oil from seeds adulterated with *Argemone mexicana* seeds, 157
- DUODENAL**, see gastro duodenal ulcer
- EDIBLES**, proximate principles of food in, by chemical methods, 315
- EL TOR** distribution of, in rural areas in India, 403, in natural water sources in absence of cholera, 419
- ELECTROCARDIOGRAPHIC FEATURES**, alterations in, by digitalis, 659
- ELEUSINE CORACANA** (millets), vitamin B₁ content of, 89
- ENDEMICITY AND EPIDEMICITY** of homogeneous cholera districts, 465
- ENZYMES** (proteolytic), competition of protein substrates towards, 655
- EPIDEMIC DROPSY**, see dropsy
- EPIDEMIOLOGY** of cholera in Bengal, 411, 425, 465, of epidemic dropsy, 157, 167, 361, 813, 839, of plague, 51
- ESSENTIAL OIL**, see *Curcuma longa*
- EXCRETION** urinary of morphine in opium addicts with and without lecithin glucose, 195, of vitamin B₆ by rats, 557, of copper in rats, 751
- EYE DISEASES** and nutrition in Bengal, 119
- FEEDING** experiments on human subjects to test toxicity of argemone oil, 839, habits of glassfishes, 851
- FILARIAL LYMPHANGITIS**, seasonal variations in, 209
- FISH** nutritive value of, 279, fish oils, vitamin D in, 699, shark and saw fish liver oils, vitamin A in, 575, Indian fish liver oils, vitamin A in, 95
- FISTULA**, see biliary
- FLEAS**, see rat-fleas
- FLIES**, see kala azar
- FLUID**, see hydrocele
- FLUORINE** poisoning in rats, 693
- FŒTAL INFECTION** by *L. icterohæmorrhagica* in a rat, 685
- FOOD** of glassfishes, 851, proximate principles of, 315
- FOODSTUFFS** metallic contamination of (with copper), 751, nicotinic acid in, 125
- FRACTIONS**, see protein
- FUNGI**, ringworm, cultures of, 691
- FUNGISTATIC** and **FUNGICIDAL** effects of substances, 23
- GASTRO DUODENAL ULCER**, experimental production of, 311

GLASSFISHES (*Ambassis* Cuv and Val.), food and feeding habits of, and their bearing on biological control of guinea worm and malaria, 851

GLUCOSE, *see* lecithin glucose

GROUND WATER pollution in alkaline alluvium soil, 887

GROUPING, *see* blood

GUINEA PIG, *see* tubercular affections

GUINEA WORM, *see* glassfishes

HÆMAGGLUTINOGENS M and N, antigenic difference between, 547 *See* also isohæmagglutination

HÆMATOLOGICAL studies in Indians, 375, 903

HÆMOLYSIS and lecithin, 773

HÆMOLYTIC *STREPTOCOCCI* in parturient women, 491, in throat, its incidence and causal relationship to puerperal infection, 483

HEXAMINE, bacteriostatic effect of human bile after oral administration of, in a patient with biliary fistula, 271

HYDATID disease, complement-fixation in, 203

HYDERABAD DECCAN, normal polynuclear count at, 225

HYDROCELE FLUID, protein fractions and other physical properties of, 253

ICTEROHÆMORRHAGIÆ, *see* *Leptospira*

INDIA *V. cholerae* and El Tor type strains in rural areas in, 403 SOUTH INDIA peptic ulcer in, 311, 665 INDIAN children, dental caries in, 709, dietaries (typical), protein, Ca and P metabolism with, 105, fish liver oils, vitamin A content of, 95, men (normal), blood urea clearance in, 627 INDIANS hæmatological studies in, 375, 903

INFANTILE MORTALITY in beriberi area in Madras, 703

INFECTION *see* pneumococcus, puerperal, fetal, *W. bancrofti* INFECTIVE larvae of *W. bancrofti*, 393

INOCULATION TEST (guinea-pig) for diagnosis of human tubercular affections, 531

INTRACELLULAR atage of *L. chameleonis*, 811

ISOHÆMAGGLUTINATION, titration of, 231

JHARIA COAL FIELDS (Bihar), dietary and physique of mining population in, 143

KALA AZAR transmission of, 783, 789, relation between malaria and kala azar in a rural area, 789, 'blocked' flies, 783

LABORATORY TESTS on fungistatic and fungicidal effects of subatances, 29

LARVÆ, *see* *W. bancrofti*

LECITHIN, and hæmolyais, 773 liver glucose treatment of opium addicts, 195

LEISHMANIA *chameleonis*, Wenyon 1921, intracellular atage of, 811, *donovani*, transmission of, by *P. argentipes*, 799, *tropica*, transmission of, by bite of *P. papatasi*, 803

LEPROSY, LEPROUS SERA, *see* complement-fixation

LEPTOSPIRA ICTEROHÆMORRHAGIÆ in a rat, fetal infection by, 685

LIVER damage, experimental, and ærum phosphatase, 647, liver damaged dogs, chloral clearance in blood in, 639 LIVER OILS, *see* fish

LONGA, *see* *Curcuma longa*

LYMPH, *slaphylococci* in vaccine, 259

LYMPHANGITIS, filarial, seasonal variations in, 209

MADRAS PRESIDENCY, infantile mortality in beriberi area of, 703

MALARIA glassfishes as control for, 85, and kala azar, 789

MAN blood urea clearance in normal Indian men, 627, myiasis producing diptera in, 883

MANNOSE, preparation of, 1

MEDICAL STUDENTS (Bombay), vital capacity of, 723

MEDIUM, PRESERVING, for transmission of specimens for isolation of *V. cholerae*, 681

METABOLISM basal, in Bombay, 287, protein, Ca and P, with typical Indian dietaries 105 METABOLIC EXPERIMENTS on absorption and excretion of copper in rats, 751

METALLIC CONTAMINATION of foodstuffs, copper, 751

MEXICANA, *Argemone*, seeds of, adulterated with mustard oil, 157

MICE, *see* pneumococcal infections

MILK, human, vitamin B₁ content of, 567

MILLETS, *see* *Eleusine coracana* and *Sorghum vulgare*

MINING POPULATION in Jharia coal fields, dietary and physique of, 143

MORPHINE, urinary excretion of, in opium addicts, 195

MORTALITY, *see* infantile

MOST PROBABLE NUMBER OF ORGANISMS by dilution tests, 499, 511

MUSTARD OIL, *see* epidemic dropsy

MYIASIS, *see* diptera

- NATURAL WATER SOURCES**, vibrio El Tor in, in absence of cholera, 419
- NERVE TISSUE**, excess, elimination of, from antirabic vaccine, 303
- NEUROTOXIN**, separation of, from crude cobra venom, 367
- NICOTINIC ACID** in blood, 133, 341, 585, in urine, 341, in animal tissues, 585, in foodstuffs, 125, in biological materials, 325, effect of washing and cooking on, in raw and parboiled rice, 83
- NIGHT BLINDNESS**, clinical, dark adaptation tests in 351
- NOMENCLATURE**, see isohæmagglutination
- NORMAL** dogs, chloral clearance in blood in, 639, Indians, blood urea clearance in, 627, Indian women in Calcutta, hæmatology in, 375, polynuclear count at Hyderabad, 225
- NUTRITION** and its bearing on preventable blindness and eye diseases in Bengal, 119, human, 105, in fish, 279 See also diet, foods, fish, milk, nicotinic acid, peptic ulcer, vitamin
- OIL** argemone, toxicity of, 813, 839, biological test of specific toxin in, 167, epidemic dropsy associated with mustard oil adulterated with *Argemone mexicana* seeds, 157, fish and fish-liver oils, vitamin A in, 95, 575, vitamin D in, 699, essential oil of *Curcuma longa*, pharmacology of, 769, toxic, isolation of active substances from, 361
- OPIUM** addicts, urinary excretion of morphine in, with and without lecithin glucose treatment, 195
- ORGANISMS**, see dilution tests, most probable number
- OXIDATION** of vitamin C, influence of pyrophosphate on, 71
- PAPATASII**, *Phlebotomus*, transmission of *L. donovani* by bite of, 803
- PARATHYROIDES** in rats fed on a poor rice diet, pathological changes in, 137
- PARTURIENT** women, hæmolytic streptococci in, 491
- PATHOLOGICAL CHANGES**, see parathyroids
- PEPTIC** digest broth for *Cl. tetani* toxin, 689, ulcer in South India, 311, 665
- PEPTONE**, preparation of, 541
- PHARMACOLOGY** of essential oil of *Curcuma longa*, 769, of *Rauwolfia serpentina*, 763
- PHLEBOTOMUS** argentipes, 799, papatasii, 803
- PHOSPHATASE** (serum), and liver and biliary damage, 647
- PHOSPHORUS** metabolism with typical Indian diets, 105
- PHYSICAL** development of Punjabi boys, 613, measurements of Bombay medical students, 723, methods of estimating vitamin A in fish oils, 575, properties of hydrocele fluid, 253.
- PHYSIQUE** of mining population in Jharia coal fields, 143
- PLAGUE**, epidemiology of, 51 See also rat-fleas
- PNEUMOCOCCUS** infections (type I), in mice, 2 sulphanilamido 4-methylthiazole against, 265
- POISONING**, see fluorine
- POLLUTION**, see ground water
- POLYNUCLEAR** count at Hyderabad, 225
- PRESERVING MEDIUM** for *V. cholerae*, 681
- PRICE JONES' CURVE**, standard, for Indian population, 903
- PROTEIN** fractions of hydrocele fluid, 253, metabolism with typical Indian diets, 105, substrates, competition of, towards proteolytic enzymes, 655
- PROTEOLYTIC ENZYMES**, competition of protein towards, 655
- PROXIMATE PRINCIPLES** of food by chemical methods, 315
- PUERPERAL INFECTION**, hæmolytic streptococci in human throat and relation to, 483
- PUNJABI BOYS**, physical development of, 613
- PYROPHOSPHATE**, influence of, on oxidation of vitamin C, 71
- RAT** absorption and excretion of copper in, 751, calcium intake and fluorine poisoning in, 693, fed on a poor rice diet, pathological changes in parathyroids in, 137, foetal infection by *L. icterohæmorrhagiae* in, 685, urinary excretion of vitamin B₁ by, 557
- RAT-FLEAS** in Calcutta and epidemiology of plague, 51
- RAUWOLFIA SERPENTINA**, pharmacology of alkaloids of, 763
- RED CELL DIAMETER**, measurement of, 903
- REDUCING AGENTS**, action of, on neurotoxin from cobra venom, 367
- RICE** and berberi, 551, effect of washing and cooking on nicotinic acid of raw and parboiled, 83, pathological changes in parathyroids in rats fed on a poor, 137, stored underground, vitamin B₁ in, 89
- RINGWORM** fungi, method of growing pure cultures of, 691
- RURAL AREA** malaria and kala azar in, 789, *W. bancrofti* in, 677, *V. cholerae* and El Tor type strains in, 403

- SANDFLIES, *see P. argentipes*
- SAW FISH liver oils, vitamin A in, 375
- SEASONAL VARIATIONS in malarial lymphangitis, 209
- SERA, leprosy, complement fixation by, 523
- SERPENTIN, *see Rauwolfia*
- SERUM PHOSPHATASE, experimental liver and biliary damage and, 647
- SHARK liver oils, vitamin A in, 375
- SKIN, mode of escape and penetration of by infective larvæ of *B. bancrofti*, 393
- SNAKE, *see cobra*
- SODIUM CHLORIDE for determination of flow of soil water, 891
- SOIL WATER, *see sodium chloride*
- SORGHUM VULGARE (millet), vitamin B, content of, 89
- STANDARD ERROR of the most probable number of organisms by dilution tests, 499
- STAPHYLOCOCCI in vaccine lymph, 259
- STATISTICS, *see cholera*, dilution tests, vital capacity
- STRAINS, *see El Tor*
- STREPTOCOCCI, hæmolytic in parturient women, 491, in throat and relation to puerperal infection, 483
- STUDENTS, *see medical students*
- SUBSTRATES, *see protein*
- SULPHANILAMIDE bacteriostatic effect of human bile after oral administration of, in a patient with biliary fistula, 271, in pneumococcus infections in mice, 265
- TECHNIQUE of grouping blood, 231
- TETANI, *see Cl tetani* toxin.
- THROAT, hæmolytic streptococci in, 483
- THYROIDS, *see parathyroids*
- TISSUE animal, nicotinic acid in, 585, nerve, elimination of excess, from antirabic vaccine, 303.
- TITRATION, *see isohæmagglutinins*
- TOXIN *Cl tetani*, peptic digest broth for formation of, 689 *See also epidemic drowsy, argemone oil.*
- TRANSMISSION *see kala azar, malaria, L tropica V cholerae*
- TROPICAL, *see L tropica*
- TUBERCULAR affections, human, reliability of guinea pig inoculation test for diagnosis of 331
- ULCER *see peptic ulcer*
- UREA *see blood urea*
- URINARY EXCRETION, *see morphine, vitamin B,*
- URINE nicotinic acid in, 341
- VACCINE antirabic, elimination of excess nerve tissue from, 303, lymph, staphylococci in, 259
- VENOM, crude cobra, separation of neurotoxin from, 367
- VIBRIO CHOLERÆ distribution of, in rural areas in India, 403, El Tor in natural water sources in absence of cholera, 419 preserving medium for, 681 *See also cholera*
- VITAL CAPACITY of Bombay medical students, 723
- VITAMIN A content of Indian fish liver oils, 95-575, deficiency amongst Bengalees, 591, determination of minimal and optimal requirements of, by visual adaptation in dark, 591
- VITAMIN B₁ content, of human milk, 567, of millets, whole wheat and rice, 89
- VITAMIN B₂ urinary excretion of, by rats, 557, 561
- VITAMIN C influence of pyrophosphate on oxidation of, 71
- VITAMIN D content of fish oils, 699
- VULGARE, *see Sorghum vulgare*
- WATER pollution of ground water in alkaline alluvium soil, 887, sodium chloride for determination of flow of soil water, 891, vibrio El Tor in natural sources of, 419
- WHEAT (whole), vitamin B₁ content of, 89
- WITEBSKY, KLINGENSTEIN and KUHN (W K K) antigen, preparation and use of, 527
- WOMEN hæmatological studies in normal Indian, in Calcutta, 375 hæmolytic streptococci in parturient, 491
- WUCHERERIA BANCROFTI infection in a rural area, 677, infective larvæ of, their mode of escape and penetration of skin, 393

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